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RESISTENCIA A ESTRESES BIÓTICOS Y ABIÓTICOS EN AVENA

DOCTORANDO

JAVIER SÁNCHEZ MARTÍN

DIRECTORES

DIEGO RUBIALES OLMEDO

ELENA PRATS PÉREZ

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TITULO: *RESISTENCIA A ESTRESES BIÓTICOS Y ABIÓTICOS EN AVENA*

AUTOR: *JAVIER SÁNCHEZ MARTÍN*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

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PhD THESIS

RESISTANCE TO BIOTIC AND ABIOTIC STRESSES IN AVENA

PhD STUDENT

JAVIER SÁNCHEZ MARTÍN

SUPERVISORS

DIEGO RUBIALES OLMEDO

ELENA PRATS PÉREZ

Julio 2012

D. Diego Rubiales Olmedo, Dr. Ingeniero Agrónomo por la Universidad de Córdoba y Profesor de Investigación del Instituto de Agricultura Sostenible del CSIC (IAS-CSIC), Córdoba.

Dña. Elena Prats Pérez, Dra. Ingeniero Agrónomo por la Universidad de Córdoba y Científico Titular del Instituto de Agricultura Sostenible del CSIC (IAS-CSIC), Córdoba.

CERTIFICAN

Que el trabajo " **RESISTENCIA A ESTRESES BIÓTICOS Y ABIÓTICOS EN AVENA**" realizado Javier Sánchez Martín 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.

Fdo: Diego Rubiales Olmedo Elena Prats Pérez

Córdoba, 29 de Junio 2012

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A mi hermano Raúl,

caminero y motero.

SUMMARY

Oat is an important grain and forage crop cultivated over than 9 million hectares globally. However, drought and biotrophic fungal diseases, such as crown rust (*Puccinia coronata* f. sp. *avenae*) and powdery mildew (*Blumeria graminis* f. sp. *avenae*) seriously threaten oat production. To combat this, agricultural scientists must develop a comprehensive understanding of the mechanisms through which plants can tolerate/resist stress and translate this to crop breeding programmes. Thus, based on a germplasm collection consisting on 141 oat landraces collected from across Southern Spain and 36 commercial cultivars we aimed to sought and characterise new sources of resistance to both, powdery mildew and rust and drought tolerance and further understand the resistance responses to generate tools that could aid the breeding of this crop.

In chapter one, entitled "Resistance to powdery mildew (*Blumeria graminis* f.sp. *avenae*) in oat seedlings and adult plants" new sources of resistance to this fungus have been identified and characterize. In addition a detailed work on plant adult resistance was performed revealing the histological bases of the resistance responses. In chapter two, "Identification and characterization of sources of resistance in *Avena sativa*, *A. byzantina* and *A. strigosa* germplasm against a pathotype of *Puccinia coronata* f.sp. *avenae* with virulence against the Pc94 resistance gene*"* new sources of resistance to a new highly virulent isolate that defeated the resistance gene *Pc94* used until now, have been identified and characterized. Both work highlight the importance of using resistance based on several responses and if possible not associated with monogenically controlled traits. To ease in the breeding of this complex traits in chapter three, entitled "QTL's association with mechanisms of resistance to oat crown rust (*Puccinia coronata* f. sp. *avenae)* in Ogle/TAM O-301 mapping population" we explored the possibility to link QTLs with specific resistance responses assessed both, macroscopically and microscopically, and successfully found several QTLs associated with resistance responses related to basal resistance. In addition, recent work of the group showed that HR-mediated resistance provokes in barley stomatal dysfunctions which could be an important component of the disease resistance cost. In chapter four, entitled "Impact of hypersensitive response elicited by different life style biotrophic fungi on oat physiology under single and overlapped biotic and abiotic stresses" we report a fitness cost associated with the execution of resistance mechanisms against *Blumeria graminis* f. sp. *avenae* and *Puccinia coronata* f.sp. *avenae*; and revealed its causes. Regarding drought tolerance, chapter five, entitled "Targeting sources of drought tolerance within an *Avena* spp collection through multivariate approaches", we identified and characterised new sources of drought tolerance in the oat germplams collection. In addition, we employed a multivariate approach to reveal the key physiological mechanism/s in the oats for coping with drought stress. Furthermore, in chapter six entitled "An integrated mechanism of drought tolerance in oats (*Avena sativa*) revealed through metabolomic analyses", we used a metabolomic approach to reveal novel drought-induced changes based in two of the oat cultivars, Flega and Patones, previously characterized as most susceptible and tolerant to drought respectively in the chapter five. These include 1) maintenance of high relative water content by salicylate-induced regulation of stomatal movements 2) specific photoprotection mechanisms to deal with the increasing oxidative stress promoted by the low CO2 concentration consequence of the partial stomatal closure. Finally, in chapter seven, entitled "Identification and multi-environment validation of adaptation of *Avena sativa* cultivars to Mediterranean environments" we characterize those accessions with most stable resistance and good performance over different Mediterranean environments using a GGE biplot analysis.

RESUMEN

La avena es un importante cultivo de grano y forraje con un área cultivada de más de 9 millones de hectáreas en el mundo. Sin embargo, la sequía y enfermedades causadas por hongos biotrófos, tales como la roya de la hoja (*Puccinia coronata* f.sp. *avenae*) y el oídio (*Blumeria graminis* f. sp. *avenae*), mitigan fuertemente su producción. Para combatirlo, los mejoradores deben adquirir una comprensión completa de los mecanismos mediante los que las plantas pueden tolerar/resistir el estrés y emplearla en los programas de mejora. Para ello, usando una colección de germoplasma de 141 genotipos silvestres recolectados a lo largo del sur de España y 36 variedades comerciales, nos propusimos encontrar nuevas fuentes de resistencia al oídio y a la roya así como tolerancia a la sequía para más tarde profundizar en las respuestas de resistencia generando herramientas que puedan ser empleadas en los programas de mejora.

En el capítulo primero, titulado "Powdery mildew resistance in oat" se han encontrado y caracterizado nuevas fuentes de resistencia frente al oidio. Además, se realizó un detallado estudio de la resistencia en planta adulta revelando las bases histológicas de las respuestas de resistencias. En el capítulo segundo, "Oat resistance against crown rust virulent on *Pc94*", nuevas fuentes de resistencia a este nuevo y altamente virulento aislado que ha superado la resistencia proporcionada por el gen *Pc94,* han sido identificadas y caracterizdas. Ambos trabajos destacan la importancia del uso de resistencia basada en varias respuestas y si es posible no asociada con rasgos monogénicamente heredados. Para facilitar la mejora de esos complejos rasgos, el capítulo tres, titulado "QTL´s associtation with mechanims of resistance to oat crown rust (*Puccinia coronata* f.sp *avenae*) in Ogle/TAM O-301 mapping population" estudiamos la posibilidad de asociar QTLs con mecanismos de resistencia específicos, macro microscópicos, y encontrar varios QTLs asociados a resistencia basal. Además recientes trabajos de nuestro grupo, ponen de manifiesto que la resistencia mediada por HR provoca disfunciones estomales en cebada que podrían ser un importante componente del coste de resistencia a la enfermedad. En el capítulo cuatro, titulado "Impact of the hypersensitive response elicited by different life style biotrophic fungi on oat physiology under single and overlapped biotic and abiotic stresses", ponemos de manifiesto un coste asociado a la ejecución de los mecanismos de resistencia frente a *Blumeria graminis* f. sp. *avenae* and *Puccinia coronata* f.sp. *avenae;* así como sus causas. Respecto a la tolerancia a la sequía, el capítulo cinco titulado "Targeting sources of drought tolerance within an *Avena* spp collection through multivariates approaches", hemos identificado y caracterizado nuevas fuentes de tolerancia a la sequía en la colección de germoplasma. Además hemos empleado una aproximación multivariante con el objetivo de dilucidar el o los mecanismo(s) más importante(s) en la tolerancia a la sequía. En el capítulo sexto, titulado "An integrated mechanism of drought tolerance in oats (*Avena sativa*) revealed through metabolomic analyses", empleamos una aproximación metabolómica para revelar nuevos cambios en respuesta a la sequía en dos cultivares, Flega y Patones, previamente caracterizados como el más susceptible y el más tolerante a la sequía respectivamente en el capítulo cinco. Estos incluyen 1) mantenimiento de un elevado contenido de agua y movimientos estomáticos regulados por ácido salicílico, 2) mecanismos de fotoprotección específica que lidien con un incremento del estrés oxidativo promovido por la baja concentración de CO_2 consecuencia del cierre parcial de estomas. Finalmente, en el capítulo séptimo, titulado "Identification and multi-environment validation of adaptation of *Avena sativa* cultivars to Mediterranean environments" caracterizamos aquellas accesiones con la Resistencia más estable y mejor adaptación a lo largo de diferentes ambientes de la cuenca del Mediterráneo usando un análisis GGE biplot.

OBJECTIVES

The main objectives of the thesis are:

- Identification and characterization of novel sources of resistance to powdery mildew caused by *B. graminis* f.sp*. avenae* in oat.
- Identification and characterisation of novel sources of resistance against a pathotype of *Puccinia coronata* f.sp. *avenae* with virulence against the Pc94 resistance gene.
- Identification in a segregating population of oats derived from the cross between the cultivated oats Ogle/TAM O-301 (OT) quantitative trait loci (QTL) markers associated with resistance phenotypes, and particularly with specific resistance responses related to basal resistance previously determined histologycally.
- Evaluation of the effects and impact of resistance responses to powdery mildew and crown rust on physiological dysfunctions, determining its probable cause. Evaluate whether physiological dysfunctions are determined by the extent of HR or the life style of the pathogens.
- Identification and characterization of new sources of drought tolerance in oat.
- Explore the usefulness of multivariate analysis to reveal key physiological mechanism/s responsible for the drought tolerance response that could be use as a selection tool.
- Construction of an integrated model explaining drought tolerance in oat thorough metabolomic approaches.
- Identification of oat cultivars with the most stable resistance to powdery mildew and crown rust and with good performance along the Mediterranean Basin using a GGE biplot analysis.

General Introduction

1. GENERALITIES OF THE OAT CROP

1.1. The Oat Crop

Oats rank sixth in world cereal production statistics, following wheat, maize, rice, barley and sorghum (Bajji *et al.*, 2002).

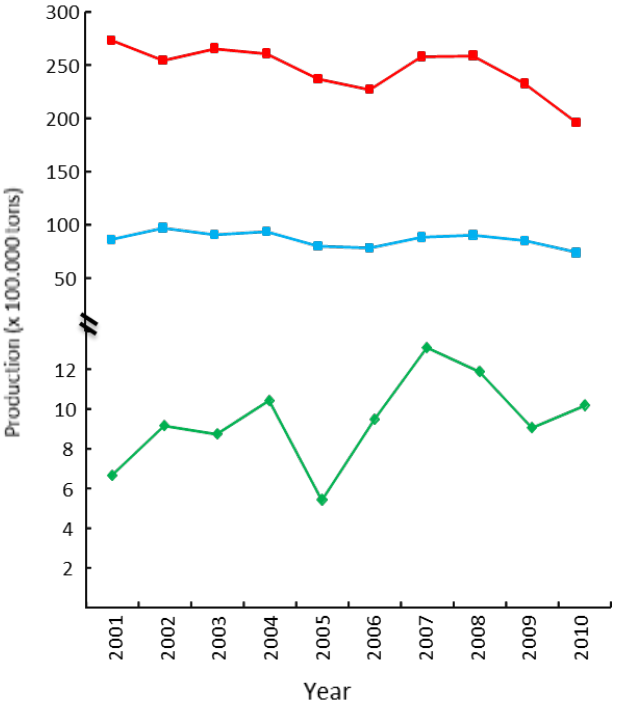


Figure 1. Evolution of the average production of oat (x100.000 tons) in the World, Europe and Spain during the last ten years (FAO, 2011).

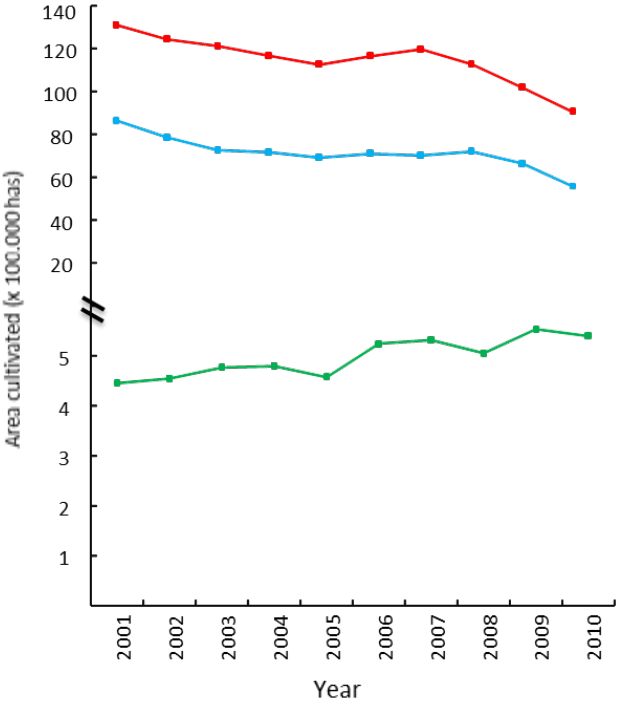


Figure 2. Evolution of oat area cultivated (x 100.000 hectares) in the World, Europe and Spain during the last ten years (FAO, 2011)

World production in 2010 was 19. 600.935 tons, 11.946.666 tons in Europe and 1.017.800 tons in Spain, the latter being the second country in European production (Fig. 1).

Oats are widely grown in temperate areas, with an increasing interest to expand the crop to subtropical areas, Mediterranean countries (Stevens *et al.*, 2004) and northeast China (Islam *et al.*, 2011). This is mainly due to its good adaptation to a wide range of soil types and because on marginal soils oats can perform better than other small-grain cereals (Stevens *et al.*, 2004).

In 2010, the oat cultivated area in the world was 9.054.956 hectares; 5.578.763 in Europe and 539.500 has in Spain being the latter the fourth country in the world and the first in Europe (Fig. 2).

1.2. Taxonomy

Oat plants are annual grasses belonging to the family Gramineae (Poaceae). There are about 70 species belonging to the genus *Avena*. The genus *Avena* brings together a complex mix of different morphological and cytogenetic groups. Within the genus *Avena* L. we distinguish three ploidy levels according to their chromosome number (Chamla, 1984) and four karyotypes (A, B, C and D).

- Diploid 2n = 14 chromosomes (*Avena strigosa*; *Avena strigosa brevis*; *Avena clauda*; *Avena longiglumis*),
- Tetraploid 2n = 28 chromosomes (*Avena strigosa* weistii; *Avena barbata*)
- Hexaploid 2n = 42 chromosomes (*Avena* sterilis; *Avena byzantina*; *Avena* fatua; *Avena sativa*).

According to (Harlan and De Wet, 1971) and (Leggett and Thomas, 1975), *Avena* spp are classified into three gene pools based on the ease of gene transfer (introgression) from the alien species into the cultivated hexaploid oats:

- The Primary Gene Pool: Given the high inter-fertility in crosses, all the hexaploid oat taxa, including the most common wild oats *A. sterilis* and *A. fatua*, were grouped into a single biological species with cultivated oats (Ladizins.G and Zohary, 1971). (Loskutov and Rines, 2011). Within this group we could achieve a good introgression of desired traits from the wild hexaploid oats by conventional crossing and backcrossing (Loskutov and Rines, 2011).
- The Secondary Gene Pool: this group includes the AACC tetraploid species *A. magna*, *A. murphyi* and *A. insularis*. Hybridizations with *A. sativa* produce highly self-sterile plants, but the F1 female fertility is enough to produce progeny. Subsequent crosses between tetraploid and hexaploid individuals are possible due to a correct pairing between chromosomes (Loskutov and Rines, 2011).
- The Tertiary Gene Pool: Defined by (Leggett and Thomas, 1995) involves all the diploid *Avena* species and the tetraploids *A. barbata*, *A. vaviloviana*, *A. abysinnica* and *A. macrostachya*. Introgression of desired traits is complex since the F_1 progeny derived from the crosses with *A. sativa* are often sterile and the development of lines free of accompanying deleterious genes is difficult.

1.3. The Origin of the Oat Crop

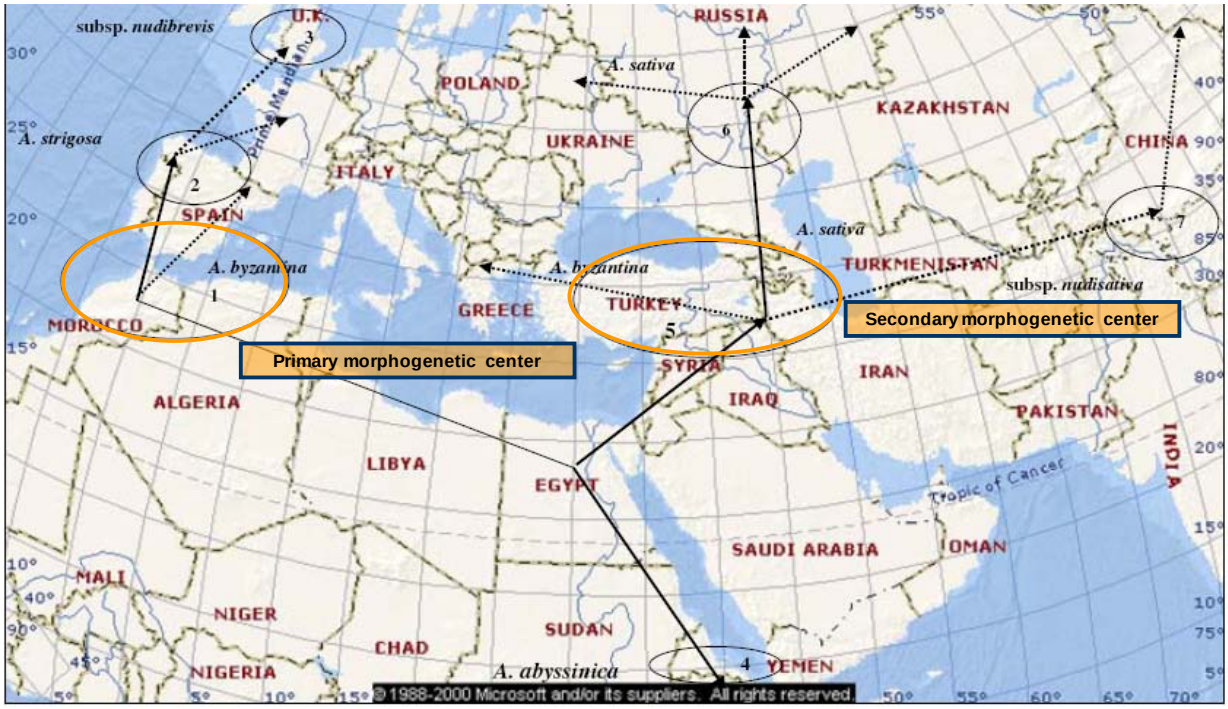
There is a great controversy about which is the center of origin and domestication of the oat crop. However, one thing seems clear, the domestication of the oat crop happened much later that for other cereals, such as barley and wheat. While the domestication of barley and wheat held at 12.000-8.000 years before present, oat domestication took place about 4000 years before present. This was so, because for many centuries, oats have spread as a weed in wheat and barley crops. Its constitution as crop with own identity did not occur until it was shifted to the north of the continent, where meteorological conditions are characterized by increased humidity and cold.

A good review of evolution and domestication of *Avena* species is summarized in (Loskutov, 2008). To date, it was accepted that the origin of *Avena* genus was the Fertile Crescent and its spread as weed within wheat and barley crop towards the rest of Europe finalized when the constitution of the oat crop by itself.

We must differentiate between the origin of the genus *Avena* L. and the center of origin and diversification of cultivated oat. According to the analyses in the global diversity of local varieties available in the Vavilov Institute of Plant Industry (VIR) oat collection, the most likely center of origin of the genus *Avena* L. lies in the western part of the Mediterranean region, while the secondary center and origin of cultivated oat (*A. sativa* L.) is situated within the Asia Minor center (Loskutov, 2008).

The first morphogenetic center of oats, the Mediterranean center, was placed between Morocco, Algeria and Spain (Fig.3). From here, the center of diversity for the diploid species A. *strigosa* was placed in Spain and Portugal towards Great Britain where naked forms designated as *A. nuda* appeared.

The second morphogenetic center of oat is placed in the Fertile Crescent, the origin and domestication center of cultivated oat (Fig. 3). From this South-West Asian Center (Turkey, Iran, Iraq and Syria) the hull-less forms (*A. sativa* subsp. *nudisativa*) were developed in Mongolia and China and *A. sativa* covar. *volgensis* in Tatarstan region.



1. Mediterranean centre – Morocco, Algeria, Spain
2. Spain and Portugal – centre of diversity of *A. strigosa*
3. Great Britain – centre of diversity of *A. strigosa* subsp. *nudibrevis*
4. Abyssinian centre – Ethiopia, centre of diversity of *A. abyssinica*
5. South-West Asian centre – Turkey, Iran, Iraq, Syria
6. Tatarstan, Bashkortostan – diversity of *A. sativa* convar. *volgensis*
7. China, Mongolia – centre of diversity of *A. sativa* subsp. *nudisativa*

..... ▶ - pathways of distribution of cultivated species and forms

Figure 3. Evolution pathways of cultivated *Avena* species. adapted from (Loskutov, 2008)

1.4. Morphology

The cultivated oat is an annual grass. The stem is composed of a series of nodes (solid) and internodes (hollow in the maturity but solid during vegetative development). The leaves are solitary, alternate, two-ranked and sessile. Each leaf is composed of a sheath, which surrounds the culm, a ligule inserted at the apex of the sheath, and a leaf blade.

Unlike the other grasses that have spikes, the inflorescence of oats is a panicle, equilateral (with spikelets arranged on both sides) or unilateral (all on one side) (Fig. 4a). This is a highly branched inflorescence with a main axis called rachis, from which nodes arise other alternating lateral axes. Both the main axis and each lateral axes, culminate in a terminal spikelets, which is the individual floral unit.

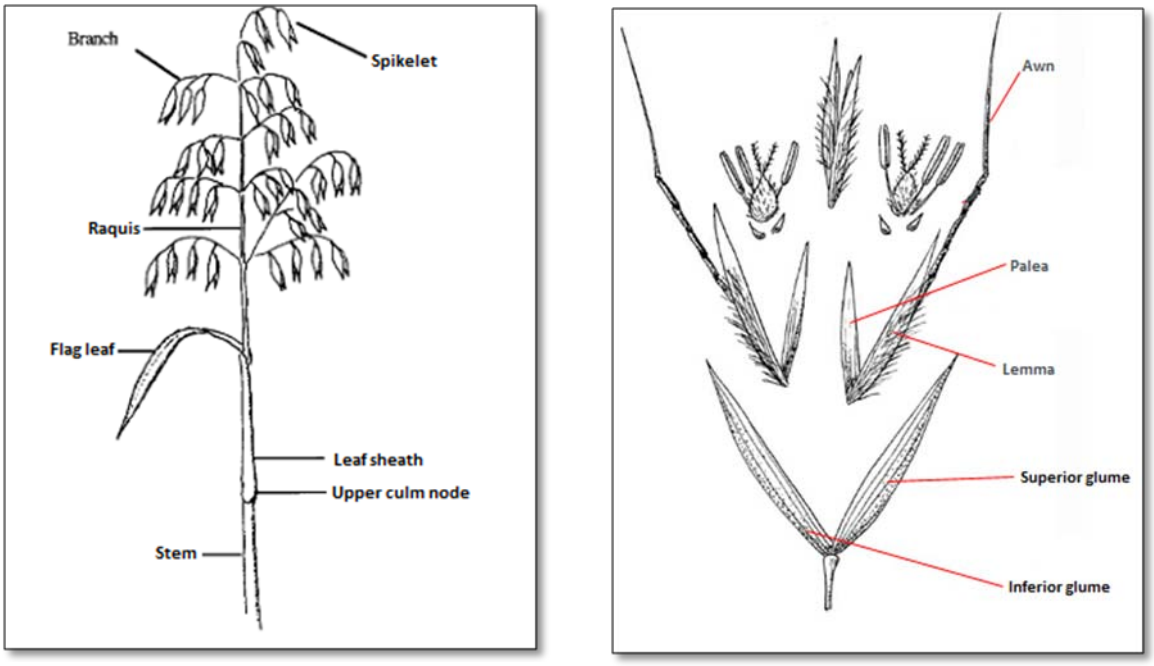


Figure 4. Oat inflorescence. A) Characteristics of a bilateral panicle and B) Spikelet of oat laid open, showing superior and inferior glume, lemma, palea and a dorsal awn.

Each spikelet contains 2-3 flowers. The first two structures within the spikelet correspond to a pair of squamous glumes at the base which includes the rest of the floral unit. The flower itself is composed of the reproductive organs (ovary and three stamens) and two bracts, a lower, lemma, and an upper palea (Fig. 4b).

1.5. Uses of the oat

1.5.1. Human food

Oats have numerous uses as human food. It is used as flakes, flour and meal. It is fairly low in gluten, so it is not suitable for making bread, although is suitable for use into oat-cakes. It is present at breakfast as cereal porridge and in special varieties of bread {FAO, 2001).

The nutritional value of oat protein is determined by three characteristics: concentration, amino acid balance, and digestibility. Among cereals, oat has the highest protein concentration, around 20%. The amino acid balance reflects the protein quality. Cereals are considered to have a scarce lysine, methionine, threonine, isoleucine and tryptophan content. However, oat protein is higher in lysine than the other cereals (Peterson, 1992). Oat protein is nearly equivalent in quality to soybean protein, which World Health Organization research has shown is equal to meat, milk, and egg protein (Chamla, 1984). In addition, oats seeds also contain all the B vitamins, vitamin E, and nine minerals, Ca, Cu, K, Mg, Mn, Na, P, Zn and high levels of Fe.

The average soluble fiber content in the oat grain is approximately 6%. Oat fibers have unique properties which contribute to the reduction of LDL-cholesterol and in turn maintain the high density HDL leading to 10 to 15% reduction of the disease cardiovascular risk. The fibers may also regulate glucose levels. Studies indicate that eating daily oats can be beneficial against colon cancer.

The lipid content of oat varieties is higher than that of other cereals. The composition is favorable for the health because of the high proportion of unsaturated fatty acids. Oat is high in linoleic acid, essential fatty acid for human nutrient (Peterson, 1992).

1.5.2. Use of whole-crop oats for animals

Oats have a lower nutritive value than cereals in general, and cannot meet the energy requirements of high-yielding animals. However, the high content of water-soluble carbohydrate in the whole plant means that oats can be used for whole-plant use for grazing, green forage, silage or hay, as well as for grain. They are often used in mixture with legumes for forage production. The straw is important bedding for livestock, as well as good roughage (FAO 2011).

Grazing or harvesting at an earlier stage, before stem elongation, allow the crop to recover and produce grain for harvest. That is a really an interesting goal, because in this early moment, the protein content is high and fiber is low, so the nutritive value is high and could be used as fodder crop (FAO 2011).

Under unfavorable years, forage oats, with longer growing season, provide farmers with the possibility of obtaining forage by harvesting areas destined for grain. For example, in Portugal and Spain, some oats are grazed in winter and thereafter are harvested for grain or hay (FAO 2011).

2. BIOTIC STRESSES AFECTING OATS

Oats, as all plants, under both natural and agronomic conditions, are subjected to stresses; external conditions adversely affect growth, development or productivity of the plants. These stresses are classified in two groups depending on its origin: biotic stress when they are it is caused by the action of an organism or abiotic stress when it's caused by a physical or chemical agent (Azcón-Bieto and Talón, 2008). Biotic stresses can be produced by animals, other plants (allelopathy) and microorganisms such as bacteria, fungi and other plant pathogens causing disease. In this work we will focus in oat diseases mainly those caused by powdery mildew and rust. Oat diseases may cause direct damages and reduction of the fodder yield or produce indirect damages, compromising the quality of the product when they produce toxins in the grains and make them unsuitable for consumption either by animals or humans (FAO 2011).

2.1. Diseases caused by FUNGI

2.1.1. Rust

Rust diseases are the most harmful diseases affecting cereals, and particularly oat. Rusts are obligate biotrophic fungi with a complex life cycle, belonging to Puccinia genus. They are heteroecious and macrocyclic. To complete their life cycle, 5 types of spores and 2 different hosts are needed. On oats two spore stages of rust can occur, telial and uredial stages. The rest of spore stages to complete the life cycle must happen in the alternative host. Symptoms can appear in all aerial green parts of the plant (leaf sheaths, panicles, even floral structures) but especially in the leaves in the case of crown rust, and the stem in the stem rust. Although plants can be infected from seedling, is at the time of maturation and flowering when the disease usually impact in the crop.

- **Crown Rust**

Crown rust, caused by *Puccinia coronate* f. sp. *avenae* is the most harmful disease affecting oats, causing high losses in yield and grain quality worldwide (Simons, 1985) particularly in the Mediterranean basin (Singh *et al.*, 1992) where populations are more virulent than in the center and north of Europe. Infection by the pathogen induces several structural, biochemical and physiological changes in its host. The more profound changes are brought about by intracellular invasion by the fungus and the formation of haustoria (Harder and Haber, 1992). Also, localized changes in photosynthesis and leaf-gas exchange were reported (Scholes and Rolfe, 1996). Following inoculation of oat leaves with crown rust, the rate of whole-leaf gas exchange declined during the sporulation stage and photosynthesis was severely inhibited over the entire leaf (REF). This disease may reduce yield up to 40%. Its development is more rapid and harmful when weather conditions allows good oat crop growth and pathogen development, this is humidity and a range of temperature between 15-25 ° C.

Life Cycle

The life cycle explained here for *P. coronate* is valid for *P. graminis*, except for the sexual part. For *P. coronate* development, the alternative host is *Rhamnus* ssp. for *P. graminis* it is *Berberis* ssp. At the end of the planting season, coinciding with the lack of nutrients and the onset of oat senescence, uredia transform into telia that produce teliospores (Fig 5 ,A), a diploid-dark and thick-walled spore resistant to adverse environmental conditions that act as latent stage for the fungus. When environmental conditions are favorable, the germination of teliospores and subsequent meiosis results in the formation of four haploid spores, basidiospores (Fig 5. B), that is not able to infect the telial host and furthermore must travel to the aecial host, where its germination produce a haploid colony called pycnia that can be of two or more mating types. Inside pycniospores are produced (Fig 5, D). This sexual recombination results in a higher frequency of new physiological races (Dhanda *et al.*, 2004) particularly during summer in the Mediterranean region, where several species of *Rhamnus*, the alternate *P. coronata* host, are widespread (Vavilov, 1992)

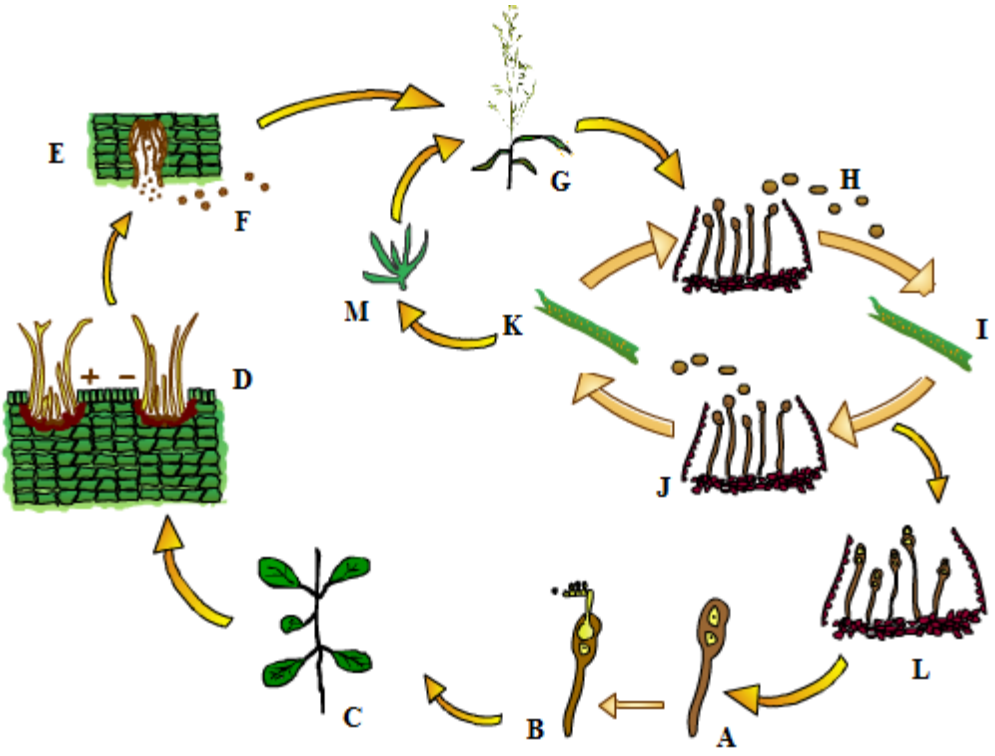


Figure5. Life cycle of *Puccinia coronata* f.sp. *avenae* A) Teliospores in telia. Under favourable conditions, teliospores germination form basidiospores (B) that travel to the aecial host, *Rahmnus* spp. (C) in where a haploid colony called pycnia is formed with pycnidiospores inside (D). The combination of opposite matin types develops a dikariotic colony which produces aeciospores (F) that travel until the telial host (G). During the spring the cyclic infection in the host (H-J) increase epidemic disease until the end of the summer when the plant finished and a resistance form of the fungus, the telia (L) is formed. The infection of the telial host could occurs from volunteers plants in the spring (M).

The distribution of pycnidiospores carried out by insects or surface moisture distributes spores on the host surface facilitating the union of the opposite mating types and resulting in a dikariotic hyphae that profilate to form an aecial colony in the abaxial surface where dikaryotic aeciospores are produced (Fig. 5F). These aeciospores travel until the telial host and germinate (Fig. 5G).

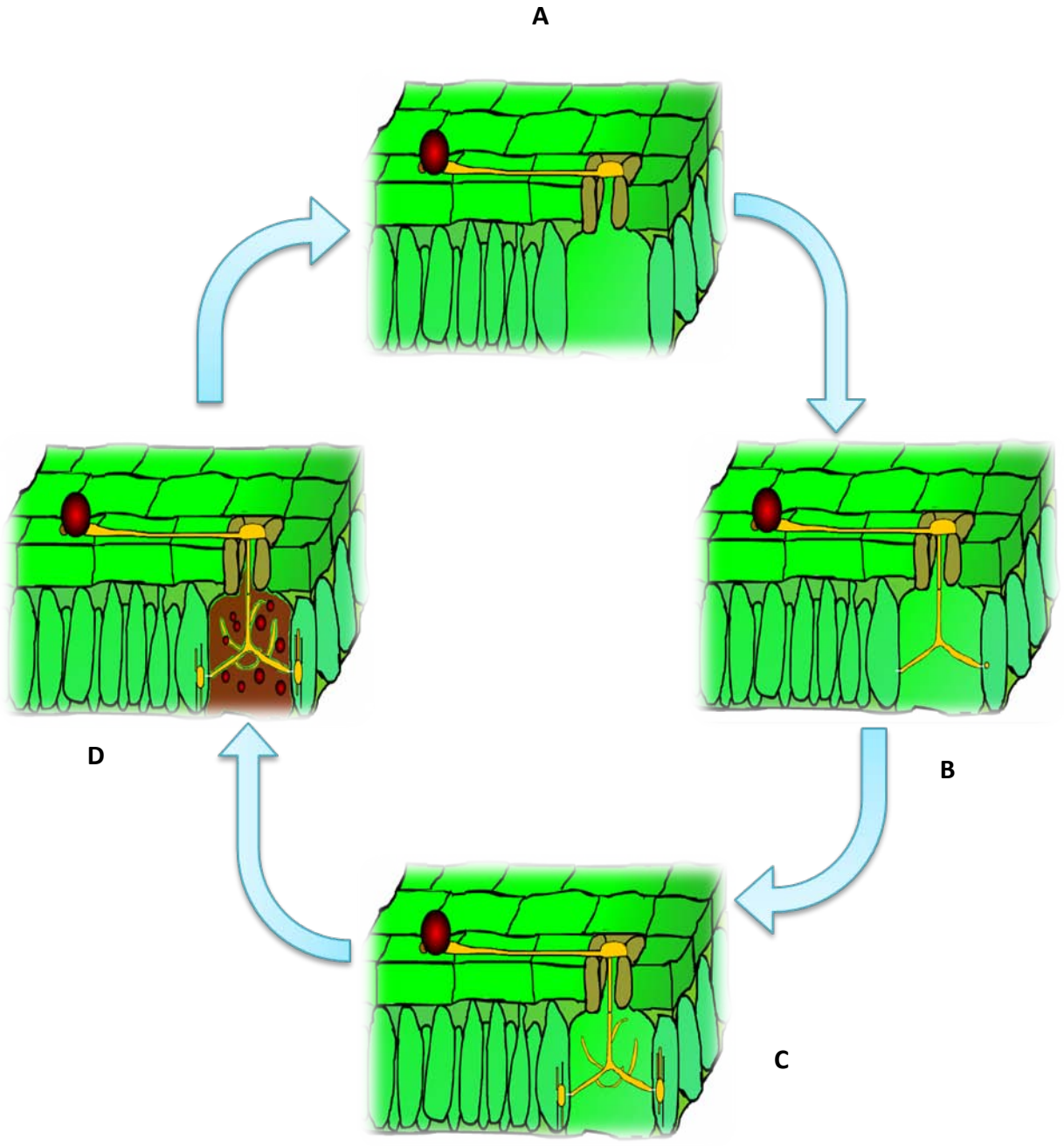


Figure 6. Infection process of oat crown rust. A) Appressorium formed over a stomata. B) Mesophyll cell penetration and starting of a haustorium formation. C) A well-established colony growing supported by the intracellular feeding structures, the haustoria. D) Colony producing uredospores to its widespread.

After a uredospore lands on the leaf surface, its germination develops an appressorial germ tube. The germ tube growths towards stomata along the leaf surface guided by chemical and physical features of the host surface (Hoch and Staples, 1987). When finding a stomata, growth of the tube ceases and an appressorium is produced over stomata in response to some physical components of the stomatal structure (Hoch and Staples, 1987) (Fig. 6A). Beneath the appressorium, a penetration peg grows reaching the substomatal cavity, a cigar-shaped substomatal vesicle (SSV) is formed, from which a secondary hypha and a haustorium mother cell, at its tip, forms (Fig. 6B). Following contact between the haustorium mother cell and the mesophyll cell, an infection peg develops, penetrates the mesophyll cell and forms a feeding structure, the haustorium, which takes up nutrients for fungal growth (Parlevliet and Kievit, 1986) (Fig 6C). The haustorium is separated from the plant cytoplasm by an extension of the plant plasma membrane and is not truly intracellular but functions as the feeding organ of the fungus (Heath and Skalamera, 1997). As a result, a colony grows and develops pustules called uredinia, containing urediniospores (Fig. 6D).

Disease symptoms consist in yellow pustules containing masses of urediospores that are exposed after the rupture of the epidermis (Fig. 7). Lesions are circular or oblong and occur on both surfaces of the leaf and can reach other green parts of the plant, when the epidemic becomes more severe. After some weeks, the borders of the pustules can turn black, with teliospores formation. When the infected plants reach maturity, production of urediospores ceases and they are then replaced by teliospores.

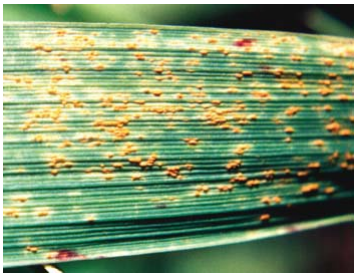


Figure 7. Pustules of crown rust on leaves caused by *Puccinia coronata* f. sp. *avenae*

- **Stem rust**

Stem rust is another harmful disease affecting oat that occurs wherever oats are grown. It is caused by *Puccinia graminis* f. sp. *avenae* and, although it could be a more destructive pathogen than crown rust fungi under favored weather conditions, the damages caused by stem rust are lower than with other rust diseases. However, these damages may seriously affect yield and quality of grain.

Disease symptoms appear on the stems and leaf sheaths like masses of urediospores called pustules (uredia), larger than those crown rust, oval or elongated, and dark-brown in color. They may appear in both surfaces on the leaf and the rupture of the epidermis expose masses of reddish-brown spores. The fungus can produce several times this part of its life cycle until plant approach maturity, when teliospores transform into telia.

2.1.2. Powdery mildew

Although powdery mildew is considered to be a major disease of wheat and barley, in the case of oats, this consideration is restricted only in those areas with a cold and humid climate. In these regions, powdery mildew is the most important foliar pathogen of oats causing annual grain yield and total biomass losses (Harder and Haber, 1992). (Jones, 1977) reported that powdery mildew caused 11 to 40% oat grain yield losses depending on disease severity and cultivar. Taking into account the potential of the oat crop for fodder, it is interesting to note that a strong epidemic of the disease at the seedling stage results in 40 to 50% forage loss , albeit, the disease does not affect grain yield because later in the season stage, with the appearance of higher temperatures, the powdery mildew is arrested. Powdery mildew overwinters primarily as mycelium on volunteers and autumn-sown crops. The cleistothecia produced during late summer act as resistant forms of the fungus, being resistant to low temperatures and drying out, allowing the fungus to survive for a time in the absence of a host.

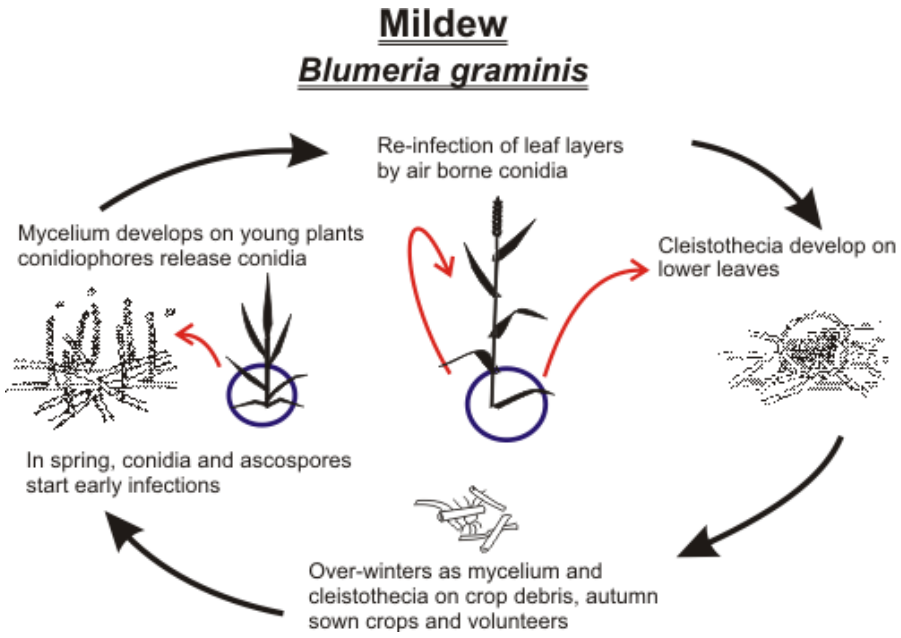


Fig 8. Life cycle of powdery mildew in oats.

Under humid conditions, cleistothecia release the sexually produced basidiospores which can initiate autumn infections. As temperatures rise in the spring, dormant mycelium starts to grow and spores are quickly produced. Basidiospores, the asexual conidia of *Blumeria graminis* f. sp. *avenae*, germinates and follows an ordered morphogenetic sequence (Green *et al.*, 2002). Following deposition on the leaf surface, a short primary germ tube (PGT) emerges after contact with the epidermal cell surface and play three main roles as a prerequisite to appressorium formation (Carver *et al.*, 2001): 1) attachment to the host surface, 2) gaining access to host water and 3) recognizing host surface features. After that, the appressorial germ tube (AGT) emerges,

elongates and differentiates a hooked, apical appressorium (Kunoh, 2002)(Fig. 9A). When a functional appressorium is formed, a penetration peg emerging, beneath the appressorium attempts to breach the plant epidermal cell wall, probably combining physical force and enzymatic degradation (Fig. 9B). The attack leads a cell response involving localized deposition of material into the inside surface of the cell wall, directly beneath the appressorium and penetration peg. These cell wall appositions, called papillae, may arrest the infection process. If this first penetration attempt fails, the fungus, may forms another lobe, usually opposite the first lobe thought nearer to the conidia, and tries the penetration again. In this way, one AGT could develop 3-4 lobes in the attempt to penetrate the cell. On susceptible hosts, penetration tends to be successful and the peg penetrates into the host cell (12-15 h.a.i) and a primary haustorium is formed (15-24 h.a.i).

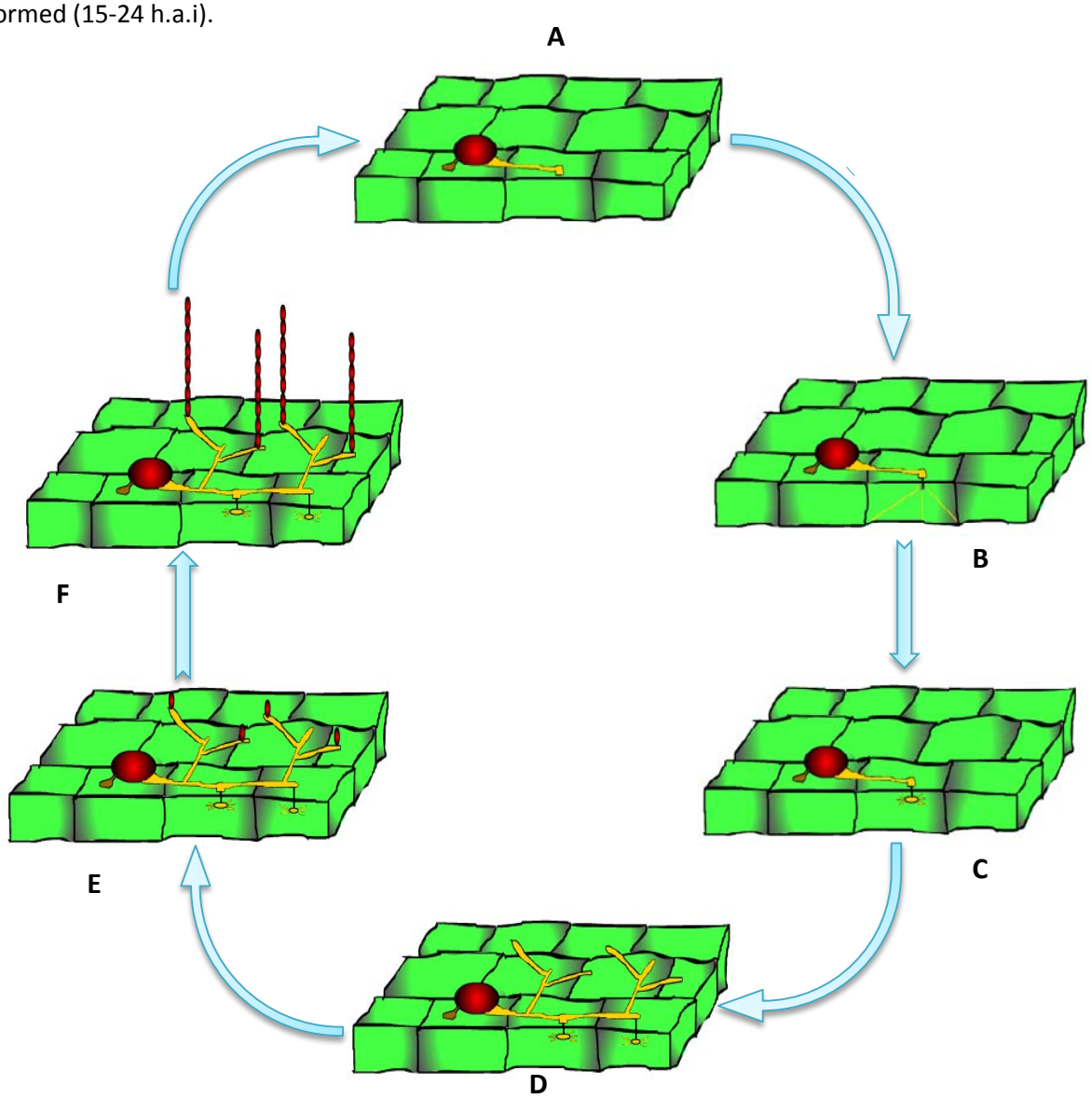


Figure 9. Infection process of *Blumeria graminis* f. sp. *avenae*, causal agent of oat powdery mildew. A) Apical hocked appressorium form on a mesophyll cell. B) Penetration peg breach mesophylls cell. C) Formation of an intracellular haustorium. D) Colony well-established. E) Beginning of formation of conidiophore and F) Chain of conidiophore

The haustorium then develops digitate processes over the next 4-5 days (Fig. 9C). This haustorium absorbs enough nutrients to feed mycelia which develops subsequent generations of haustoria and conidiophores (Fig 10, D-F). At this point, the asexual lifecycle is completed.
The disease is most destructive when mild winters are followed by dry weather in spring and early summer, particularly when late-sown crops occur near infected winter oat (Jones and Griffiths, 1952). At the end of the season, volunteers and early autumn-sown crops may become infected, providing inoculum for the following crop.
The symptoms appear as colonies with a grey-white superficial mycelium on the upper surfaces of the lower leaves of the plant, although some pustules may develop on the underside of the leaf (Fig. 10). The pustules could be surrounded by chlorosis and often join together to form large masses of fundal growth on the upper surface of leaves when conditions are favorable. At the disease progresses, the patches become grey or brown, and may eventually extended to upper areas in the plant if the weather conditions are favored. Severely diseased leaves collapse and die. Powdery mildew is most prevalent on lower leaves but can cause blighting of the upper leaves, heads and awns of susceptible cultivars.

Figure 10. Mycelium of powdery mildew on leaves caused by *Blumeria graminis* f. sp. *avenae*.

2.1.3. Pyrenophora leaf bloth

The causal agent is the fungus *Pyrenophora claetomioides*. The symptomatology is characterized by small white dots surrounded by a brown-reddish halo, which, under appropriated conditions, coalesce and expand, forming small longitudinal lines. On the stems, appears another symptom, called blackstem, characterized by darkening of the nodes and fragility of the stem. Seeds are affected by the fungi during flowering of the spikelet showing a darkening on the surface. Seeds may act as a reservoir for the pathogen for several years. Then, crop rotation is not a useful method of control for this disease and genetic resistance is the most desirable method of control.

Figure 11. Symptoms of Leaf blotch on oat, caused by *Pyrenophora chaetomioides.* (FAO, 2011).

2.1.4. Scab

Also called Fusarium head blight is caused by *Fusarium* spp, although the most important species is *F. graminearum*. The fungus usually attacks the seedlings, which may die. If plants survive will show later infected spikelets pale, light pink or buff colored. Grain will be light, wrinkled and wilted and contaminated with toxic compounds, which makes its use for human or animal food impossible. As there are not resistant resources in oats cultivars, crop rotation and the use of fungicides in the seeds are the only methods for the control of this disease.

2.2. Diseases caused by VIRUS: Barley Yellow Dwarf Virus

Symptoms appear as yellowish-green spots around the tips of older leaves that can coalesce. Infected plants are dwarfed, mature early, produce seed low in bushel weight, and have more empty hulls at the base of the panicle.

2.3. Diseases caused by BACTERIA: Halo Blight

The most common bacterial disease of oats is the Halo Blight, caused by *Pseudomonas coronafaciencs*. At the beginning of the disease, the lesions, mainly localized in leaves, appear as small, pale green oval spots. As the disease progresses, the affected tissue die and the spots coalesce in bigger lesions in which color change to gray or brown. The pathogen is able to survive on seeds and plant residue, being the last one, an important source of disease for next seasons. Although it's known that this disease is not a serious economic threat, under specific conditions it can seriously damage the crop.

3. Main control measures for oat diseases.

There are several methods of control against biotic stresses; however, no single practice is available to completely control all diseases. The biotic stress control should be done as part of an integrated pest management. An integrated control program is defined as the strategy that allows control of biological stress with no economic damage, using all appropriate techniques combined as possible, benefiting to the whole society. Thus the different control methods should consider not only the biological and agroecosystem factor but also related ecosystems and society that will use agricultural products (Pimentel, 1982; Kogan, 1998).

The various control methods can be classified into four categories: agronomic, chemical, biological and genetic control.

3.1. Cultural methods

3.1.1. Crop rotation

In general, avoidance of monoculture is a good technique to reduce the impact of pests, diseases and weeds. In some areas where the same crop is sown repeatedly after years, the diseases related to it are established, incremental population and the damage is more severe.

3.1.2. Escape:

Sometimes is possible coincidence between the period of maximum susceptibility of the plant and the highest abundance of the parasite through the use of early maturing cultivars or early date planting (Last, 1954). Thus, it has been reported a reduction of *P. coronata* damages in early rippening oats (Simons and Michel, 1968) and by an earlier planting date (Simons, 1966).

3.1.3. Reducing inoculum

Any cultural practice that minimizes the amount of over-wintering inoculums on volunteer plant should help to reduce disease levels of powdery mildew in oat (Harder and Haber 1992). In those areas where Rhamnus and Berberis hosts acts actively generating new sources of genetic variability for the pathogen, its eradication, through mechanical elimination or using foliar fungicides, could help reducing crown and stem rust epidemics. This method is applied in the dispersal part of the life cycle the pathogen.

3.2. Chemical control

Chemicals has been used for more than a century to control plant diseases, but acquired a special significance from the 40's with the development of broad-spectrum organic pesticides. Chemical control has been effective in controlling many pests and weeds and is still widely used and necessary in intensive agriculture for high yields. However, in many cases it has been ineffective and presents a series of problems. Thus, it should be used wisely taking into account the proportion between advantages and disadvantages, some of these related below:

- The use of chemical is very inefficient; most of them are wasted on the surface of plants and eventually end up on the soil surface, resulting in soil contamination. On the other hand pesticides also accumulate in certain vulnerable parts of the plants, such as the roots, stems or within the fruit.
- Pesticides and their residues are highly toxic to environment and human health.
- The use of inadequate doses of chemicals can lead to reduced plant vigor making it more susceptible to attack by new parasites.
- Selection pressure exerted when pesticides are used massively makes parasites rapidly acquire resistance to the component used.

Oat crop is not considered a very profitable economic cereal for a wide use of fungicides taking into account the economic costs. Even so, (Jones *et al.*, 1985) assured that a systemic fungicidal seed treatment together with an adult plant resistant could be used controlling oat powdery mildew. Systemic fungicides have been successfully used against crown rust (Rowell, 1984). However, its use has been refused on economic grounds and difficulties to have an unique and effective formulation so its use is only recommended in very susceptible varieties under weather conditions that favored the epidemic (Soovaeli and Koppel, 2011).

3.3. Biological control

Biological control consists on the use of any living organism or substances derived there from, to control biotic factors that can damage a crop. Obviously the effectiveness of biological control depends on the combination between the agent employed and the pathogen. The use of biological must take into consideration the impact that parasites or predators may have in the ecosystem and their relationship to other crops in the area (Bélanger and Labbé, 2002).

3.3.1. Powdery mildew

- **Antibiosis**: *Tilletiopsis spp*. has been reported as an antagonist who gets a good control of cucumber powdery mildew. In addition, *Pseudozyma flocculosa* has been demonstrated to be effective against *Podosphaera pannosa* and *B. graminis* f.sp. *tritici* responsible for rose and wheat powdery mildew, respectively (Bélanger and Labbé, 2002).

- **Parasitism:** since powdery mildews have mostly an ectotrophic life style, one can assume that they are easy targets for hyperparasites. However, very few organisms have been reported to rely on or invade powdery mildew structures to complete their life cycle. *Ampelomyces quisqualis* was the first organism to be reported as a hyperparasite of powdery mildew. *Verticillium lecanii* is a polyphagous fungus and it has been reported to parasitize arthropods, rust and powdery mildew (Bélanger and Labbé, 2002).

3.3.2. Rust:

- **Parasitism:** *Tuberculina* spp. is known to attack several rusts. For example, *T. persicina* attacks the rust of the asparagus (*Puccinia asparagi*) and *Puccinia cacabata* that causes rust on cotton. In addition, *Verticillium* ssp. are hyper parasites of different rust fungi. Some works have reported its parasitism against the coffee rust, carnation rust, bean rust and stem rust of wheat. Others mycoparasites of rust have been described; *Cladosporium* against sugarcane rust, *Trichoderma spp*. on stem rust of cereals (Rubiales, 2000). Bacteria haven also been reported to act as parasite against rust. For example, *Pseudomonas fluorescens* and *Bacillus megaterium* which parasite the uredospores of cereals stem rust (Rubiales, 2000).

- **Antagonist:** *Cladosporium tenuissimum* has been reported as antagonist due to its ability to inhibit in vitro aeciospore germination of the two-needle pine stem rust, *Cronartium flaccidum,* and *Peridermium pini* (Moricca *et al.*, 2001). (Haddad *et al.*, 2009) reported the biological control of coffee rust by antagonistic bacteria, *Bacillus* and *Pseudomonas spp*. under field conditions.

3.4. Genetic resistance

The most desirable method for protecting plants from diseases is the use of resistant cultivars. Developing resistant cultivars requires the sought o sources of resistant, donors that restore the diversity lost in cultivated oats but that initially possessed their wild progenitors. This highlights the importance of maintain, screen and characterise genetic resources. About 220.000 oat accessions in ex situ collections have been estimated in the state of the world's plant genetic resources report

(Loskutov and Rines, 2011). Large collections are held by the USDA (20.000 accessions), the PGRC, Canada (30.000 accessions) and within the framework of the ECP/GR (34.146 accessions), namely by the Vavilov Institute of Plant Industry (VIR, Russia) (about 12.000 accessions), which has a collection of about 10.000 accessions of four cultivated and 2.000 accessions of 21 wild species (Loskutov, 2001). In FAO/VIEWS (htpp://apps3.fao.org/views/germplasm.htm), 29 collections listed maintain accessions of wild Avena species (Table 1) (Loskutov and Rines, 2011).

Institution	Country	Number of accessions
Agriculture and Agri-Food Canada, Plant Gene Resources of Canada, Saskatoon Research Center	Canada	14.935
USDA-ARS, National Small Grains Germplasm Research Facility	USA	10.908
N.I. Vavilov Research Institute of Plant Industry	Russia	2.001
Tel-Aviv University Institute Cereal Crop Development Lieberman Germplasm Bank	Israel	1.544
Agricultural Research Center, Australian Winter Cereals Collection	Australia	549
Aegean Agricultural Research Institute, Department of Plant Genetic Resources	Turkey	311
Institute for Plant Genetics and Crop Plant Research – Genebank	Germany	300
National Wheat Research Center	Brazil	254
National Plant Genetic Resources Center Plant Breeding and Acclimatization Institute	Poland	168
Agricultural Research Organization, Volcani Center, Israel Gene Bank for Agricultural Crops	Israel	117

Table 1 Number of accessions of wild oat species in the main genebanks

Some genebanks accumulate specific and geographic diversity of wild oat species not only by means of natural collection but also through seed exchange with and ordering samples from other gene banks (Table 2)

Species	**Number of accessions**
A. Strigosa	697
A. abyssinica	615
A. barbata	2.526
A. fatua	2.341
A. sterilis	22.951

Table 2 Representation of wild Avena species in ex situ collections in the world (Germeier, 2008)

It should be noted that hexaploids species, *A. sterilis* (*A. ludoviciana*) and *A. fatua*, represent the main part of wild oat accessions in the ex situ collections because they are of great importance as breeding material and are easy to conserve and propagate in the field (Loskutov and Rines, 2011). Although acreage grown to oats is continuously declining in Europe, oat still plays an important part in the genetic resources work within the European Cooperative Programme for Plant Genetic Resources (ECPGR). This program has been established as a platform to strengthen cooperation of European ex situ collections already in 1980. The Avena Working Group has been established in 1984 as one of the original six Crop Working Groups (Germeier, 2008).
Nowadays, the European Avena Database (EADB) has passport data of 32.910 accessions representing collections from 26 European contributors and nearly 170,000 characterisation and

evaluation observation points for 3134 accessions. Besides, Avena is one of the four model crops represented in a European initiative for "An European Genebank Integration System" (AEGIS), which also represents the regional strategy for Europe.

In the last 60 years, efforts have been done in order to incorporate resistance genes against different pathogens in cereals and also oat cultivars. The use of resistant varieties has some advantages over other control methods, especially over the use of chemicals (Niks *et al.*, 1993):

- Use of resistant cultivars represents an economic saving. If we have a complete resistance, it is not necessary the use of chemicals; if the resistance is partial, the dose employed is lower.
- The seed cost is the same compared with susceptible varieties.
- Resistant varieties are safer because its use avoid the risks of using chemicals for farmers and have no potentially harmful residues.
- Contrary to what happens with chemical control, use of resistant varieties is compatible with other control methods, such as biological control.

However, the use of resistant varieties is not a perfect solution.

- Developing resistant varieties, using conventional breeding methods, is a very long process.
- Resistant varieties may be low yielding due to the namely "resistance cost" discussed later.
- Resistant varieties are usually resistant to a specific pathogen or even isolate (race-specific resistance), while there are wide spectrum chemicals that act against several pathogens.
- The resistance usually is not durable in time, although this also occurs with the use of chemicals.

4. Race-specific and wide-spectrum resistance in plant breeding.

Over the last decades there has been a continuous debate on the classification of the forms of resistance and its relation with the breeding of resistance crops.

4.1. Race-specific resistance:

It is one of the best understood forms of resistance. It is associated with elicitation of the hypersensitive response (Mur et al., 2008) (HR, discussed later) and based in the gen-for-gen interaction between an avirulent (*Avr*) gene from the pathogen and a resistance (R) gene from the plant. Due to the monogenic nature of this resistance it has been the most widely used in breeding programs for years. However, under field conditions HR is almost invariably overcame by pathogens because mutation of *Avr* genes matching the R genes is favored by selection since this new pathogenic isolates avoid recognition and plant defence (John and Dangl, 2006). In turn, new specificities of R genes may then be generated by variation of the leucine-rich repeats that they encoded in the plants.

Due to the relative easy management and despite the short durability of this form of resistance it has been widely use in oat resistance, particularly against rusts. Indeed, the use of race-specific (*Pc*) genes for rust resistance has been the primary mean of control. Currently, more than 90 genes for

crown rust resistance have been assigned with permanent designations (Chong *et al.*, 2000). For instance, from the primary gene pool, *A. sterilis*, the progenitor of cultivated oat, has been found to be quite a riche source of crown resistance genes allowing introgression of *Pc38, Pc39* and *Pc68* to develop hexaploids resistance oat cultivars (McKenzie *et al.*, 1981; McKenzie *et al.*, 1984; Brown *et al.*, 2001). However, the resistance was overcome and search for additional sources of resistance including new *A. sterilis* lines was necessary to do. Other *A. sterilis*-derived *Pc* genes for crown rust resistance include *Pc58* in TAM-0-301 (McDaniel, 1974a), *Pc59* in TAM-0-312 (McDaniel, 1974b) and finally *Pc60* and *Pc61* (Leonard and Martinelli, 2005). In addition, from the secondary gene pool, the resistance *Pc91* from *A. magna* has been used to develop the cultivar HiFi (McMullen *et al.*, 2005) and *A. strigosa* from the tertiary gene pool has been the source to introgress *the Pc15*, *Pc23* and *Pc94 genes*, to hexaploids *Avena* species (Dyck and Zillinsk.Fj, 1963; Rines *et al.*, 2007).

Although not as widely used as in rust resistance, sources of resistance to powdery mildew and specific resistance genes have been identified. The partially dominant gene *Eg-1* was found in a hexaploid oat derived from the cross between *A. sativa* and *A. ludoviciana* (Jones, 1952). Another powdery mildew resistant gene *Eg-2*, was reported in the diploid *A. strigosa* ssp. *hirtula* by the same author. Later (Hayes and Jones, 1966) reported the powdery mildew resistance gene Eg-3 traces to a wild oat, A. *sterilis* L. var. *ludoviciana* This resistance has been transferred to many oat cultivars grown commercially in Belgium, France, Germany, Great Britain and other countries (Hsam *et al.*, 1997). Gene *Eg-4* was found in a tetraploid *A. barbata* and successfully transfered to a cultivated hexaploid oat by induced translocation (Aung *et al.*, 1977) or interfering with regular meiotic behaviour using the diploid specie *A. longiglumis* (Thomas *et al.*, 1980). Recently, a new powdery mildew resistance gene from *Avena macrostachya*, *Eg-5* gene, has been successfully introgressed into hexaploid oat *Avena sativa* (Yu and Herrmann, 2006). A. sterilis has been reported as a source of resistance to oat powdery mildew (Roderick *et al.*, 2000) and although chromosomal heteromorphology between the diploid and tetraploid donors and *A. sativa* could make difficult the crosses, some successful introgression of resistance genes from *A. barbata, A. hitula, A. ventricosa, A. prostate* and *A. macrostachya* to hexaploids oat cultivars have been done (Thomas, 1968).

Unfortunately, as previously stated these genes have been usually defeated rapidly by new populations of the pathogens, because of selection pressure resulting from large-scale and long-term cultivation practices (Leonard and Martinelli, 2005; Chong and Kolmer, 1993; Leonard *et al.*, 2004, 2005a; Leonard *et al.*, 2005b). Thus, in the last year several strategies such as the use of multilines or pyramiding have been adopted to improve the durability of the resistance to crown rust. Gene pyramiding is based in the idea of combine in just one cultivar two or more single resistance genes. Thus, it would be necessary the occurrence of two or more simultaneous mutations to overcome the resistance. However, it's difficult to work with gene pyramiding due to the dominance and epistasis effect of multiple resistance genes. In addition, since two or more resistance genes may have similar reactions to numerous races it makes necessary the use of molecular markers to tag specific rust resistance genes (Chen et al., 2007). Even so, it would take few years to gather in a single variety two or more genotypes because of the backcrosses needed. Multilines are mixtures of individual varieties that are agronomically similar (precocity, flowering time, mechanical harvesting, and grain quality) but differ in their resistance. Theoretically, the varieties would be obtained by parallel backcrosses and these varieties would be isogenic lines. However, in practice, the varieties used in the mixtures have often only a common phenotypic base. The mechanism involved in the protection of the multilines, is not completely understood. It seems clear that a fewer number of susceptible plants, would reduce the amount of inoculum available for development of the epidemic. In the same way, the presence of a resistant variety in the mixture could act as mechanical barrier, difficulting

pathogen dispersion. Also, induced resistance during incompatible interactions may play a role in these mixtures. Thus, the use of multilines with *Pc* 51 and *Pc* 52 genes have been successfully used in reducing rust severity in oat (Frey, 1982).

4.2. Wide spectrum resistance

Unlike race-specific resistance, wide spectrum resistance confers an incomplete, partial but more durable protection to the pathogen. This is because it is often controlled by several genes, reason why it's called polygenic or multigenic resistance. These several genes are involved in hampering numerous steps of the infection process activating different resistance mechanisms often previous to cell penetration by the pathogen (Agrios 2005). However, due to its complex genetic base it is difficult to improve cultivars with this form of resistance, mainly due to the lack of appropriate selection tools for selecting the appropriate individuals from the segregating populations.

One of the difficulties of managing the wide spectrum resistance is the subjective assessment of the phenotypic resistance parameters. Recently, in order to avoid the subjectivity of such assessments, more objective methods based on digital image analysis (Diaz-Lago, Stuthman et al. 2003) has been used in these evaluations. Although these phenotypic approaches can estimate the heritability and the weight of dominance and additive effects in the control of the traits, these methods do not give information about the location and the number of genes involved (Fondevilla, Fernandez-Aparicio et al. 2010).

The development of genetic maps and Quantitative Trait Loci (QTL) analyses has been a major breakthrough in the characterization of quantitative traits, enabling the identification of associated genomic regions and their contribution to the phenotypic variation. In addition, the mapping of QTLs is an useful tool to identify molecular markers linked to the resistance genes that could be used to assist breeding (Fondevilla, Fernandez-Aparicio et al. 2010).

Only a limited number of QTLs for partial resistance to crown rust have been identified and they have been determined by using the impact of the disease in agronomic traits (Diaz-Lago, Stuthman et al. 2002) or macroscopic evaluation of disease symptoms such as disease severity and infection type (IT) (Zhu and Kaeppler 2003; Barbosa, Federizzi et al. 2006; Zhu, Leonard et al. 2003; Jackson, Obert et al. 2007; Acevedo, Jackson et al. 2010), hence, the specific resistance responses linked to the QTLs were not elucidated.

Currently it is commonly accepted that together with a wide genetic resource base and appropriated selection tools, understanding the mechanisms underlying the plant resistance response is crucial in order to improve cultivars for durable resistance. Thus, there has been an increasing impetus for modern breeding strategies to employ the physiological, biochemical and molecular characteristics responsible for resistance phenotypes, which may better reflect lineage productivity, coupled with a requirement to integrate responses to environmental stress into any assessment (Araus, 1996, Richards, 1996, Slafer & Araus, 1998).

5. Resistance responses of plants to pathogens

It is now clear that there are, in essence, two branches of the plant immune system. One uses transmembrane pattern recognition receptors (PRRs) that respond to slowly evolving microbial- or pathogen-associated molecular patterns (MAMPS or PAMPs), that therefore act before pathogen invade host cells. The second acts largely inside the cell, using the polymorphic NB-LRR protein products encoded by most R genes (Dangl and Jones, 2001). They are named after their characteristic nucleotide binding (NB) and leucine rich repeat (LRR) domains. Pathogen effectors from diverse kingdoms are recognized by NB-LRR proteins, and activate defence responses leading to cell death. NBLRR- mediated disease resistance is effective against pathogens that can grow only on living host tissue (obligate biotrophs), or hemibiotrophic pathogens, but not against pathogens that kill host tissue during colonization (necrotrophs) (Jones and Dangl, 2006).

The plant immune system can be represented as a four phased 'zig-zag' model (Fig. 12). In a first phase, PAMPs (or MAMPs) are recognized by receptors, resulting in PAMP-triggered immunity that can halt further colonization. This phase is also named basal resistance and may act at different stage of the infection process. In phase 2, successful pathogens deploy effectors inside the cell contributing to pathogen virulence. However if a given effector is 'specifically recognized' by one of the NB-LRR proteins, an effector-triggered immunity leading to disease resistance and, usually, a hypersensitive cell death response (HR) at the infection site is developed (phase 3). Finally, in phase 4, natural selection drives pathogens to avoid host recognition either by shedding or diversifying the recognized effector gene, or by acquiring additional effectors that suppress the previous reaction. In turn natural selection results in new R specificities so that the host effector-triggered immunity can be triggered again.

In this work we will focus on the resistance responses that plants may trigger as part of the basal resistance and the effector-triggered immunity. In addition a brief consideration will be also taken with those constitutive characteristic that may confer resistance without the induction of the immune system. Example will focus mainly on the rust and powdery mildew-plant interaction since they are the main target of our studies.

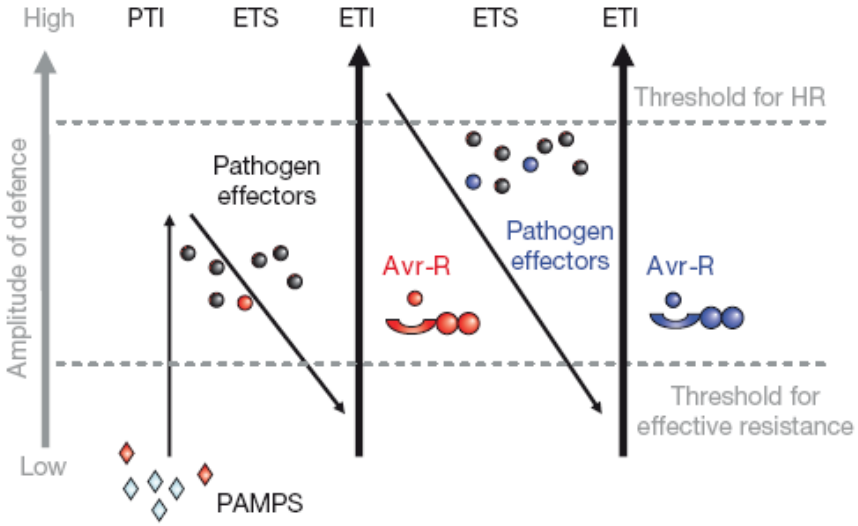


Figure 12. Zigzag model illustrating the quantitative output of the plant immune system. From Jones and Dangl, 2006

5.1. Constitutive defenses

Constitutive, physical leaf characteristics may influence pathogens germination, appressorial tube elongation, and appressorial formation. For both, powdery mildew and rusts germination adhesion of the germ tube to the leaf surface is necessary to start the infection process (Mendgen, 1978) so that features avoiding these early processes successfully contribute to plant resistance. In addition, failed stomata penetration, by germ tubes "lost" or misplaced appressoria" is responsible for the disease resistance observed in occasions in plants attacked by rusts ((Niks and Rubiales, 2002) y las referencias de dentro. (Sillero and Rubiales, 2002; Rubiales and Moral, 2004; Patto and Rubiales, 2009):

5.1.1. Waxes

Waxes on leaf surfaces may form a water repellent surface avoiding the formation of a film of water necessary to germination. Thus, low appressorium formation by various leaf rusts of cereals (*P. triticina, P. hordei, P. recondita*) in some genotypes of *Hordeum chilense* and of other wild barleys (till 10 times fold reduction) have been reported, but unfortunately not in accessions of the cultivated barley, *H. vulgare* or any cultivated cereal (Rubiales and Niks, 1992; Rubiales and Niks, 1996). In addition a marked reduction in appressorium formation by *P. hordei* on some cer-mutants (Rubiales et al., 2001) or in stomatal recognition by *P. striiformis* in some resistant wheat cvs. have been also found (Broers and Lopez-Atilano, 1996).

5.1.2. Trichomes

Trichomes have been also reported to hamper the germ tubes in reaching a suitable penetration site, considering them as a physical barrier to infection (Martin and Glover, 2007) and in *Uromyces*, this can retard germination of the surface of bean leaves by trapping the spores (Mmbaga *et al.*, 1994). Finally, (Chattopadhyay *et al.*, 2011) reported a powdery mildew resistance in field grown mulberry (*Morus* spp.) associated to high trichome density.

5.2. Basal resistance

In addition to these constitutive characteristics, PAMPs may induce a battery of mechanisms to avoid cell invasion by the pathogen.

5.2.1. Production of fungitoxic compounds:

Fungitoxic compounds excreted to leaf surface may inhibit conidia germination, and appressorium formation. Thus, in rice, inhibition of germination and appressorium differentiation by *Magnaporthe grisea* has been reported to occur, probably by the accumulation of fungitoxic leaf diffusates accumulated in resistant and partial resistant cultivars (Pasechnik *et al.*, 1997). Studies by (Prats *et al.*, 2002) demonstrated how coumarins accumulations (like scopolin, ayapin and scopoletin) on sunflower leaf surface prevents rust germination tube growth and appressorium differentiation, describing the "lost" stage of the fungus.

5.2.2. Cell-wall modifications

The cell-wall constitutes the first line of defense against fungal pathogens. It is composed of a framework of cellulose microfibrils that are embedded in a matrix of hemicelluloses, pectins and structural proteins. During the infection process, microbes produce a number of cutinases and cell wall hydrolyzing enzymes, such as pectinases, cellulases, xylanases and polygalacturonases (PGs) that attack the cell wall polymers, to breach the cell wall and enter into the plant. Plants can exhibit inhibiting proteins of these enzymes, such as polygalacturonase-inhibiting proteins (PGIPs) that inhibit PGs conferring resistance against pathogens reviewed by (De Lorenzo *et al.*, 2001). Also, plants may exhibit some cell-wall modifications leading to plant cell wall strengthening. The materials involved in the thickening of the host cell wall range from minerals (silicon, calcium and sulphur) to more or less complex organic polymers, including callose or lignin.

- **Papillae formation:**

  Fungal penetration attempts may be hampered by encasement of the penetration peg in a very localized deposition of material between the cell wall and the plasmalema, known as "papilla response" (Zeyen *et al.*, 2002). Papillae are chemically complex appositions comprising inorganic and organic constituents including callose (a carbohydrate containing β-1-3, linked glucan as the most important constituent) and autofluorogenic phenolic (Prats *et al.*, 2006). Papillae are deposited by the epidermal cell cytoplasmic aggregate onto its own inner wall surface, directly beneath the appressorium contact area (Zeyen *et al.*, 2002). Their deposition involves generation of NO (Prats *et al.*, 2005) and H_2O_2 (Vanacker *et al.*, 2000). H_2O_2 provides oxidative power necessary to drive protein cross-linking, polymerization or esterification of phenolic compounds (Zeyen *et al.*, 2002) in the papilla area.

- **Cell wall lignification:**

  It makes the cell wall more resistant to mechanical pressure applied during penetration by fungal appressorial (Vance *et al.*, 1980). Additionally, lignin plays a role as an impermeable film and thus, less accessible to cell wall-degrading enzymes. Therefore, induced lignification represents an efficiency inducible structural barrier for plant pathogens.

- **Crosslinking:**

  Protein cross-linking has been shown as a rapid and effective defensive response against intruding pathogens like bacteria, fungi or parasitic plants. Extensins and other Hydroxyproline rich glycoproteins (HRGPs), proline-rich proteins (PRPs), and glycine-rich proteins (GRPs) are structural proteins present in the cell walls. They can be rapidly insolubilized after wounding, pathogen penetration or elicitor treatment and it is a very fast response which enhances cell wall resistance within just a few minutes after pathogen attack. This process implies the formation of covalent cross-links and is mediated by H_2O_2 and peroxidases (Perez-de-Luque *et al.*, 2006).

5.3. Hypersensitive resistance (HR):

Successful pathogens that circumvent the basal resistance response usually triggered an HR like resistance response (Jones and Dangl, 2006). Then, this kind of resistance occurs after the pathogen reach the cytoplasmic content (Heath, 1981). Harold Flor first described the dependence of the HR and resistance on *R* gene-interaction with pathogen encoded avirulence (*avr*) gene production – hence the term gene-for-gene interactions (Flor, 1956). Subsequently, a large number of *R* genes have been cloned and can be broadly classified into five classes (Martin *et al.,* 2003). A near ubiquitous feature of this resistance gene products (RGP) is the possession of variable numbers of leucine-rich repeats (LRR), and frequently nucleotide binding sites (NB).Those NB containing RGP that have either regions of homology to insect Toll or mammalian IL-1 receptors- the TIR domain - form the TIR-NB-LRR *R* gene classes. Another major class of *R* gene has a coil-coil motif instead of a TIR domain and is designated CC-NB-LRR.

Among the first signs of HR are H^+ and Ca^{2+} efflux from the apoplast and, within the attacked cell, the transient generation of nitric oxide and H_2O_2 (Prats, Gay et al. 2006; ThordalChristensen, 1997). The whole cell subsequently shows autofluorescence, as phenolic compounds accumulates. Apoptotic features in oat cells at and around the infection sites were observed to various oat pathogens: oat crown rust, the halo or stripe blights of oats and the blast fungus.(Yao *et al.*, 2002). Some studies have reported the hypersensitive cell death in oat (*Avena sativa* L.) involved in resistance against crown rust (*Puccinia coronata* f. sp. *avenae*) (Tada *et al.*, 2001; Tada *et al.*, 2004) and victoria blight (Coffeen and Wolpert, 2004).

It is apparent that all nutrients necessary for elongating powdery mildew and rust secondary hyphae and colony growth must be absorbed via haustoria. Thus, in absence of hypersensitive response, restriction of haustorial development may result in limited fungal invasion. Restriction of haustorium growth may arise from several factors which include nutritional limitation or possibly physical restriction by the haustorial sheath which showed to be an invagination of the host plasmamembrane separating it from the host cell (Bracker, 1968). As the sheath appears to be derived from host tissue, host genotype could exert an influence. Thus, reduction in colony size could result from a lack of host nutrients suitable for colony growth or, as the sheath seems to behave like a semipermeable membrane through which nutrients must pass before being absorbed by the haustorium (Bushnell, 1972, Hirata, 1967), sheath structure may affect nutrient passage. Altogether, this resistance, only very poorly studied, lack for the selection pressure for pathogen change implicit in that kind of resistance causing a complete block to pathogen development such as hypersensitive response. Any restriction in size and/or efficiency of haustoria must necessarily affect such characters as latent period, sporulation capacity and colony size and so should reduce disease development. If the factors limiting colony growth can be combined with those limiting primary infection (i.e. penetration resistance), it should be possible to produce varieties with a high level of broadly based and hopefully durable resistance.

In conclusion several important factors are crucial for improving plant crops with durable resistance including availability of resistance sources, appropriate selection tools and characterisation of the resistance responses. Chapters 1 and 2 explore the possibility to identify novel resistance resources to powdery mildew and rust respectively in a germplasm collection containing landraces and cultivars and characterise the underlying resistance response leading to the resistant phenotype.

6. Disease resistance and plant fitness

As previously stated, the best approach to fight pathogens in a more sustainable agriculture is through the achievement of resistance cultivars. However care should be taken in the use of resistance to a particular pathogen in order to avoid undesired effects such as rapid apparition of new pathogen strains, susceptibility to other diseases or stresses, and yield penalties (Brown 2002; Niks and Rubiales 2002). There has been long evidence that disease resistance may affect crop performance (Smedegaard-Petersen and Tolstrup 1985) although most experiments were done a decade or two ago and have not been properly followed up (Brown 2002). This fact is important since, if resistance has a substantial cost for plants; it has a commercial significance because it may hinder the more important objective of increasing yield and quality.

The term “cost of resistance” is mainly used to explain the fact that induction of defences may be costly for the plant (reviewed in Burdon and Thrall, 2003; Heil and Baldwin 2002; Purrington, 2000; Brown, 2003)). One of the first demonstrations of the resistance cost showed that barley heavily inoculated with an avirulent isolate of *Blumeria graminis* f. sp. *hordei* had lower grain yield, smaller grains and less grain protein than the uninoculated controls (Smedegaard-Petersen and Stolen 1981). Nevertheless, this early evidence for the resistance cost have had remarkably little impact on thinking about disease resistance until quite recently (Brown 2002; Heil 2001; Heil and Baldwin 2002), Swarbrick et al., 2006). The cost necessary to induce defence and in particular the hypersensitive response has also been the explanation of the low yield increase observed in the mixtures and multilines in which individual component plants within a crop carry different R genes, despite the low disease level observed compared with a traditional crop. Altogether, few is known about the possible nature of the cost that has often been directly associated to the energy “lost” in inducing the defence but it was never related to the specific mechanism/s involved in the defence reaction and its effect in the plant physiology.

However recent work in barley (Prats et al., 2006; Prats et al., 2010) showed that HR-mediated resistance provokes in barley stomatal dysfunctions which could be an important component of the disease resistance cost. This is important since due to the monogenic nature of this resistance mechanism it has been the most widely used in breeding programs for years. This might be therefore an important contributor of the yield penalty observed in field in plants apparently healthy, such as in the mixtures and multilines (Prats et al. 2007a). The importance of this phenomenon justify further consideration and the work performed in Chapter 4 “Impact of hypersensitive response elicited by different life style biotrophic fungi on oat physiology under single and overlapped biotic and abiotic stresses”.

7. Abiotic Stresses

Abiotic stresses are the primary cause of crop loss worldwide, reducing average yields for most major crop plants by more than 50% (Bray EA, Bailey-Serres J et al. 2000). Abiotic stresses may have a physical nature: water stress (deficit or excess), temperature (high or low), salinity and UV radiation, or a chemical origin; heavy metals, toxins and alteration of the mineral components are the most representative.

However, among all of the resources necessary for plant growth and development, water can be considered the most important and limiting factor. In fact, currently drought is one of the main constrains preventing crops plants from expressing their full genetic potential (Farooq et al., 2009) so

that in the present work, we will focus on drought stress. The effects of drought in plants range from morphological to molecular levels and are evident at all phenological stages of plant growth at whatever stage the water deficit takes place. The knowledge of the impact of drought on plants is important since differences in the drought effect on the plants together with changes in the resistance responses may indicate drought tolerance and may be used as marker for selection. This is extensively studied in Chapter 6, "Targeting sources of drought tolerance within an *Avena* spp collection through multivariate approaches".

7.1. Effects of drought on plants

7.1.1. Effects on crop growth and yield.

Cell enlargement, differentiation and division are key phenomena involved in the plant growth. All of them are strongly influenced by water deficit since water is one of the physical forces involved in the cell enlargement. Impaired mitosis, cell elongation and expansion result in reduced plant height, leaf area and crop growth under drought conditions. Drought-induced yield reduction has been reported in many crop species by reduction the number of tillers, spikes or grains per plant. Moreover, the flower production and grain filling is hampered (Taiz and Zeiger, 2006). Decline in the rate of grain growth resulted from reduce sucrose synthase activity, while cessation of growth resulted from inactivation of adenosine diphosphate-glucose-pyrophosphorylase in water-stressed wheat (Ahmadi and Baker, 2001).

7.1.2. Assimilation partitioning.

Drought stress frequently enhances allocation of dry matters to the roots, which can enhance water uptake due to a preferential accumulation of starch and dry matter in roots as an adaptation to drought (Desouza and Dasilva, 1987). Drought stress decreases the photosynthetic rate and disrupts the carbohydrate metabolism and level of sucrose in leaves that spills over to a decrease export rate. Limited photosynthesis and sucrose export to the sink organs and ultimately affect the reproductive development (Kim *et al.*, 2000).

7.1.3. Nutrients

Drought stress reduces the availability, uptake, translocation and metabolism of nutrients. A reduced transpiration rate due to water deficit reduces the nutrient absorption and efficiency of their utilization (Farooq et al., 2009). Therefore, water stress affects plant mineral nutrition and disrupts ion homeostasis. It is difficult to identify the effects of drought on mineral uptake and accumulation in plant organs but in general, moisture stress induces an increase in N, a decline in P and no effects on K. Briefly four main ions are affected by drought stress; calcium, potassium, phosphorous and nitrogen.

Calcium: under drought conditions, a decrease in Ca_2^+ is reported. Calcium is involved in structural and functional integrity of plasma membrane and other cellular structures. Thus,

decreses in calcium levels might affect the stability and integrity of these cell structures. For example, in moderate and severe stressed leaves of bean, Ca_2^+ content has been reported to be low (Dogan and Akinci, 2011) although some reports indicated that water stress favored increases in this element in maize (Tanguilig *et al.*, 1987) an barley (Nambiar, 1977).

Potassium: is reported as a mineral nutrient playing a key role in osmotic adjustment and stomatal movement. K^+ deficient plants have lower resistance to water stress. An increase of this mineral nutrient has reported in tolerant varieties of wheat ( )

Nitrogen: Influence of drought on plant nutrition may also be related to limited availability of energy for assimilation of NO_3^-/NH_4^+. Besides, its uptake is hampered under water stress. For instance, nitrogen uptake decreased in soybean plants under water stress conditions (*et al.*, 1987) and nitrogen deficiency causes cotton plants to be sensitive to stress (Singh and Gupta, 1993)

Phosphorus: P and PO_4^{3-} contents in the plant tissues diminished under drought due to lower mobility of PO_4^{3-} in low moisture soils ( ).

7.1.4. Water relations

Under drought stress, some important characteristics of the plant such as relative water content (RWC), leaf water potential, stomatal resistance, rate of transpiration, leaf temperature and canopy temperature are influenced. Exposure of plants to drought led to noticeable decreases in leaf water potential and RWC with an increase in leaf temperature in wheat plants (Siddique *et al.*, 2000). Change in leaf temperature may be an important factor in controlling water leaf status under drought stress. Canopy temperature, obtained by infrared thermometry, was proposed as a crop water indicator by (Jackson *et al.*, 1981) and widely used in drought studies since then. Also, numerous studies have associated increased stomatal resistance (or its opposite, diminution of stomatal conductance) with response to water deficit in cereals (Otoole and Cruz, 1980). As previously reported (Medrano *et al.*, 2002), stomata close in response to drought before any change in leaf water content was detectable. This is attributed to the abscisic acid (ABA) root-to-leaf signaling promoted as the soil dries. The stomatal closure is followed by parallel decreases of net photosynthesis.

7.1.5. Photosynthesis

Photosynthesis is particularly sensitive to water deficit. In accordance with a decrease in relative water content (RWC) and leaf water potential, there is a decrease in photosynthetic rate (Lawlor and Cornic, 2002). However there are controversial reports about whether the origin of the photosynthetic decrease is the result of stomatal closure or a direct metabolic impairment (Tezara *et al.*, 1999; Sharkey, 1990). It´s evident that stomata close progressively with increased drought stress. Thus, water deficiency reduces the supply of carbon dioxide and photosynthetic carbon assimilation in favor of photorespiration. In addition, metabolic impairment may also contribute to photosynthetic rate decrease since: 1) under drought conditions, reduction in chloroplast volume may lead to desiccation within the chloroplast, which in turn leads to conformational changes in rubisco (Reddy *et al.*, 2004), furthermore, acidification of chloroplast stroma due to drought conditions inhibits rubisco activity (Vu *et al.*, 1987); 2) Water stress reduces activity of other photosynthetic enzymes such as phosphoenolpyruvate carboxylase, nicotinamide adenine dinucleotide phosphate-malic enzyme, fructose-1,6-bisphosphatase and

pyruvate orthophosphate dikinase; 3) Inhibition of chlorophyll biosynthesis; 4) Impaired adenosine triphosphate synthesis (Tezara *et al.*, 1999)

7.1.6. Oxidative damage

As stomata close during progressive drought stress, an imbalance between excess incident light and the CO_2 available for reduction lead to reactive oxygen species generation (Foyer and Noctor, 2000). In fact, under conditions of photon excess, cyclic electron flow either through photosystem (PS)I or PSII to down regulate quantum yield. However, if there is more excitation than can be processed, photoinhibition occurs and highly reactive species are produced. In PSII highly reactive singlet oxygen can be produced via triplet chlorophyll P680 (Asada 2006, Krieger-Liszkay, 2004). In PSI, under low $NADP^+$ concentrations, Fd will reduce O_2 instead of FNR yielding O_2^-. O_2^- dismutates to H_2O_2 and dioxygen, in a reaction catalysed by superoxide dismutase (Cruz et al., 2004). Increase of ROS, if not properly scavenge lead a denaturation of functional and structural macromolecules, including DNA, protein and lipids causing oxidative damage an impairing the normal functions of cells, overall enhancing peroxidation of membrane lipids, degradation of nucleic acids and both structural and functional proteins.

7.2. Mechanisms of adaptation to drought stress

Several morphological, physiological and molecular plant responses can contribute for coping with drought stress. These may be classified in three groups (Turner *et al.*, 2001): shortening of the crop duration to complete life cycle before stress, **escape**; increasing its ability to avoid damage, **avoidance mechanisms** and/or to maintenance of metabolic functions under water limiting conditions, **tolerance mechanisms**

7.2.1. Escape

Plants may complete the life cycle before the onset of severe drought by different ways:

- **Early flowering** time is probably the most representative trait associated with drought escape. Drought escape occurs when plant phenological development is successfully matched with periods of soil moisture availability avoiding stress period that could mitigate plant yield (Araus et al., 2002). Developing varieties that mature before the onset of severe terminal drought has successfully increased the yield crop under drought-prone conditions (Kumar and Abbo, 2001). However, we must be careful reducing crop duration, because yield is correlated with it and an excessive decline in crop duration under favorable conditions could mitigate optimum yield (Turner et al., 2001). Specifically in oats, using *Avena barbata*, (Sherrard and Maherali, 2006) found selection for earlier flowering under drought conditions.

- **Early vigor**

  It is considered one of the most import drought escape mechanism. Early vigour should be combined with appropriate phenology for the target environment. Correlation between initial growth vigour and other characterises in recombinant imbred lines of chickpea showed that high growth vigour had significant negative correlation with days to first flower,

flowering, first pod and maturity (Toker, 2006). In cultivated oat early planting, rapid germination and early emergence results in a competitive advantage over wild oat having a great effect of final yield (Willenborg et al., 2005). In fact, the selection of oat genotypes with larger seeds suitable for sowing in areas displaying moisture stress will help to reduce the risk of poor stand establishment and will enable more homogenous growth under varying rainfall conditions (Mut and Akay, 2010).

7.2.2. Avoidance

It is the ability to avoid damage by keeping the water content high in the plant tissues. This may be achieved by:

- Closing stomata, reducing light absorbance through rolled leaves (Ehleringer and Cooper, 1992), dense trichome layer increasing reflectance (Larcher, 2000), steep leaf angles, decreasing canopy leaf area through reduced growth. Others traits are shedding of older leaves, production of smaller leaves, with more densely distributed stomata and leaves covered by epicuticular waxes or thicker cuticles.
- Maximising water uptake developing an adapted root system to drought conditions. Root thickness and root dry weight per tiller have been found to be implicated in drought avoidance (Champoux *et al.*, 1995). In fact, all root parameters could be considered as drought tolerance traits: biomass, length, density, depth, branching, diameter, and root to shoot ratio (Gowda *et al.*, 2011; Bray EA *et al.*, 2000).
- Storing water on below ground organs

7.2.3. Tolerance mechanism

Understanding plant tolerance to drought is of fundamental importance and forms one of the major research topics in plants research. Osmotic adjustment, osmoprotection, antioxidation and a scavenging defense system have been the most important bases responsible for drought tolerance to counteract the previously state drought induced damages. However, the physiological basis of genetic variation in drought response is not clear; in part, because highly complex mechanisms are likely involved. Some of these mechanisms are described below (Farooq *et al.*, 2009).

- **At physiological level**

  - ✓ Maintenance of high tissue water potential despite a high soil water deficit. Mechanisms such as improved water uptake under stress and the capacity of plant cells to hold acquired water, reduce water loss and water storage are important. Plants respond to water deficit using mechanisms as improved root traits (Price *et al.*, 2002) and by reducing water loss through reduced epidermal (stomatal and cuticular) conductance, reduced radiation absorption, and reduced evaporative surface (leaf area).
  - ✓ Maintenance of turgor pressure through osmolite accumulation and cell wall elasticity. (Clifford *et al.*, 1998) reported that changes in cell wall rigidity are important in drought tolerance in
  - ✓ Cell membrane stability. Biological membranes are the first target of many abiotic stress (Levitt, 1980). It's generally accepted that the maintenance of integrity and stability of

membrane under water stress is a major component of drought tolerance in plants (Bajji *et al.*, 2002); indeed cell membrane stability is a physiological trait widely used for the evaluation of drought tolerance in wheat (Singh *et al.*, 1992; Dhanda *et al.*, 2004; Blum and Ebercon, 1981) and rice (Agarie *et al.*, 1995). The causes of membrane disruption are unknown; it supposed that a decrease in cellular volume causes crowding and increases the viscosity of cytoplasmic components. This increases the chances of molecular interactions that can cause protein denaturation and membrane fusion (Farooq *et al.*, 2009). Thus, the adaptation of oat seedlings to water stress has been associated with changes in the lipid composition of the plasma membrane of root cells increasing permeability for glucose and decreased permeability for protons as compared to control vesicles and highlighting the importance of the ratio phosphatidylcholine (PC) / phosphatidylethanolamine (PE), the levels of cerebrosides and free sterols and the possible interaction of these components for the plasma membrane in that acclimation (Berglund *et al.*, 2004).

All that previously state for improving disease resistance crops, can be applied for improving drought tolerance crops, including availability of resistance sources, appropriate selection tools and characterisation of the resistance responses. In Chapter 5 "Targeting sources of drought tolerance within an *Avena* spp collection through multivariate approaches**"** we identified drought tolerance sources in a germplasm collection containing landraces and cultivars characterising the physiological responses during a water deficit time course. Furthermore, taking into advantage the different variables studied we performed a multivariate study to test the potential of this approach for selecting drought tolerant plants in segregating populations.

- **At biochemistry level**

Tolerance of environmental stress such drought arises from integration of events occurring at molecular and biochemical levels which are manifested at the physiological and morphological level previously seen. At the molecular level drought is perceives as signal that brings about changes in expression of genes and synthesis and modification of protein activity. At biochemical level drought tolerance responses are related with profound metabolism changes mostly leading to osmotic compound production. In this work we will focus in the biochemical responses which is a reflection of further molecular changes.

- ✓ **Acumulation of osmotic compounds.**

Compatible solutes and osmotic adjustment through overproduction of compatible organic solutes (Serraj and Sinclair, 2002); low-molecular-weight, highly soluble compounds contribute not only to osmotic adjustment, but also contributes to the detoxification of ROS, stabilization of membranes and native structures of enzymes and proteins. This involve accumulation of specific compounds such as sugars (i.e. from the raffinose family oligosaccharides, RFO) sugar alcohols (such as mannitol), amino acids (such as proline) and amines (such as glycine, betaine and polyamines) which allows the cell to decrease osmotic potential and hence increase the gradient for water influx and turgor. Thus, osmotic adjustment has been related to grain yield under water deficit environments (Moinuddin *et al.*, 2005) and it is considered as a selection criterion for drought tolerance in wheat (Peterson, 1992).

- Sugar and sugar alcohols. Galactinol and raffinose function as osmoprotectans during drought stress. Trehalose is a non-reducing disaccharide of glucose that functions as a compatible solute in the stabilization of biological structures under abiotic stress (Garg *et al.*, 2002). Trehalose enhance drought tolerance by stabilization of dehydrated enzymes, proteins and lipid membrane, as well as protection of biological structures under desiccation stress rather than regulation water potential (reviewed by (Farooq *et al.*, 2009). Fructans, besides being a reserve carbohydrate, is accumulated in drought tolerant plants (Kerepesi and Galiba, 2000). It is not clear its action mode, maybe, protecting membranes or other cellular components from the adverse effects of drought. For example, (Vereyken *et al.*, 2003), reported a role of fructans in the membrane lipid organization in dry state. Although the accumulation of the sugar alcohol mannitol has been reported to have a role as osmolite in some transgenic models plants like tobacco (Tarczynski *et al.*, 1993), its accumulation under drought conditions in cereals seems to have a more protecting role, by scavenging hydroxyl radicals and/or by stabilization of macromolecular structure (Seki *et al.*, 2007) (Abebe *et al.*, 2003).

- Aminoacids. Accumulation of proline is one of the first plant responses to water stress. Proline has widely been studied because its accumulation provides drought tolerance in many species. It is thought to play a multifunctional role to cope water stress; participates in cellular homeostasis, including redox balance and energy status. Also, it acts as signaling molecule to modulate mitochondrial functions, influence cell proliferation or cell death and trigger specific gene expression which can be essential for plant recovery (Szabados and Savoure, 2010). Furthemore, proline also contributes to enzyme and membrane integrity, scavenging free radicals and buffering cellular redox potential under stress conditions (Ashraf and Foolad, 2007).

- Citrulline is a non-protein amino acid recently described as a hydroxyl radical scavenge involved in watermelon drought tolerance protecting DNA and enzymes from oxidative injuries (Akashi et al., 2001).

- γ-aminobutyric is another non-protein amino acid, playing a role not only as an osmotic regulator but also detoxifying ROS, converting putrescine into proline and involved in the intracellular signal transduction (Kinnersley and Turano, 2000).

- Amines

  - Polyamines (PAs) such as putrescine, spermidine and spermine are accumulated under abiotic stress providing tolerance in many species such as rice or wheat (Erdei *et al.*, 1990; Yang *et al.*, 2007) playing as osmolites increasing leaf water potential.
  - Glycine betaine is thought to protect plants by maintaining the water balance between the plant cell and the environment, and by stabilizing the structure and activity of macromolecules (Sakamoto and Murata, 2002).

✓ **Increasing antioxidant metabolism:**

Under drought there is an increase of the production of ROS that might cause oxidative damage numerous in the plants cell. This is alleviated by the join action of both enzymatic and non-enzymatic antioxidants systems. Among antioxidant enzymes are catalases, superoxide dismutase, peroxidases, ascorbate peroxidases, glutathione reductase and monodehydroascorbate reductase. The non-enzymatic antioxidant systems include β-carotenes, ascorbic acid, α-tocopherol, reduced glutathione (REF).

Most of the previously described drought tolerance mechanisms have been inferred from model plants. However, despite the apparent success of stress research on these model plants, the findings have been rarely applied to improved crops. One of the key reasons relates to the genetic and physiological differences between the model and crop species (Skirycz et al., 2011). In addition those also studied in a wider number of species showed that even when substantial similarities in the tolerance responses among crops exist, there are also significant differences. For instance, physiological responses leading to increased leaf water potential are among the main responses of barley or chickpea to increasing water deficit (Matin et al. 1989; Pannu et al. 1993); however, it is not a defining feature of tolerance in bread wheat (Schonfeld et al. 1988) or faba bean (Ricciardi et al. 2001). This make necessary to explore the most determinant tolerance responses for a given species. However, the small information available on databases for non-model crops has often limited a deeper research on these. Metabolomic studies to describe large scale metabolic changes due to a particular event may circumvent the lack of database information and then it is highly appropriate for using in commercial crops. Indeed, metabolomic is currently acknowledged as highly useful for dissecting key routes involved on the interaction of plant and environment (Messerli et al. 2007). In chapter6 “An integrated mechanism of drought tolerance in oats (*Avena sativa*) is revealed through metabolomic analyses” we carried out a metabolomic based study to elucidate an integrated mechanism of drought tolerance in oats.

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Resistance to powdery mildew (*Blumeria graminis* f.sp. *avenae*) in oat seedlings and adult plants

J. Sánchez-Martín, D. Rubiales and E. Prats*

CSIC, Institute for Sustainable Agriculture, Apdo. 4084, E-14080 Córdoba, Spain

In this work, 165 *Avena sativa* and *Avena byzantina* accessions were screened for resistance to powdery mildew caused by *Blumeria graminis* f.sp. *avenae* and the defence mechanisms of resistant plants were further characterized. Ten resistant and moderately resistant accessions were selected according to macroscopic assessment. A detailed histological study of selected genotypes showed a range of defence mechanisms, acting alone or in combination, that impeded fungal development at different stages. Since the resistance observed in the collection was scarce, a study of adult plant resistance was carried out in 45 genotypes selected from field trials. Nine oat landraces and two commercial varieties showed very high levels of adult plant resistance. A detailed study of the components of the adult plant resistance revealed a high increase of penetration and post-haustorial resistance in the fifth compared to the first leaves. Identification of the resistance sources and characterization of underlying defence mechanisms will be useful for future breeding programmes and for further cellular and molecular studies to unravel the genetic basis of resistance, in this species in particular and in cereal–powdery mildew interactions in general.

Keywords: adult plant resistance, *Avena*, hypersensitive response, oat, papilla, penetration resistance, posthaustorial resistance, powdery mildew

Introduction

Oat fungal diseases are major constrains for this crop. Among the pathogenic fungi infecting oats, biotrophic pathogens such as the powdery mildew agent *Blumeria graminis* f.sp. *avenae*, with very efficient mechanisms of spread, are the most difficult to control by means of crop management, such as rotation. The use of resistant cultivars is one of the best alternatives to control these pathogens, since it avoids the use of expensive chemicals that threaten consumers and the environment (Stevens *et al.*, 2004). However, the resistance obtained is often overcome by emerging pathogenic races. This is mainly caused by the inappropriate use of resistance sources, of monogenic nature. Thus, it is necessary to identify novel sources of resistance and, furthermore, characterize the specific resistance responses/mechanisms at cellular and molecular levels. In breeding terms this may allow the combination of responses acting during different fungal developmental stages and the use of mechanisms with a polygenic base, as well as aiding the selection process (Prats *et al.*, 2007).

*E-mail: elena.prats@ias.csic.es

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Blumeria graminis f.sp. *avenae* is an obligate biotroph whose asexual conidia germinate and develop reasonably synchronously through a highly ordered morphogenetic sequence (reviewed by Green *et al.*, 2002). Emergence of a short primary germ tube is followed by that of the second, appressorial germ tube that elongates and differentiates a hooked, apical appressorium within about 12 h. A penetration peg emerging beneath the appressorium attempts to breach the plant epidermal cell wall, probably combining physical force and enzymatic degradation. If successful, it enters the cell lumen where its tip swells and differentiates into a mature haustorium that develops numerous digitate processes over the next 4–5 days. This absorbs nutrients from the epidermal cell to support growth of ectophytic secondary hyphae, from which are formed subsequent generations of haustoria (from 3 days) and conidiophores (from 4 days).

Two of the mechanisms by which cereals defend against powdery mildew are by forming papillae – cell wall appositions deposited on the inner surface of epidermal cell walls directly beneath appressoria – and by the death of attacked cells. Attacked cells that form an effective papilla survive, and much evidence indicates that papillae present a physical and/or chemical penetration barrier (reviewed by Zeyen *et al.*, 2002). Failure of attempted penetration, as a result of plant resistance associated with papilla formation and/or cell wall strengthening, obviously prevents colony establishment. Papillae have a callose matrix containing various inorganic and organic constituents including proteins and

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autofluorescent phenolic compounds (Zeyen *et al.*, 2002). Papilla effectiveness may relate to the rate of deposition, to quantitative or qualitative characteristics, or a combination of these factors.

In addition, death of the invaded epidermal cell after penetration of the host wall/papilla barrier arrests haustorium development and again stops colony development. In barley, interaction with an avirulent powdery mildew isolate is associated to some extent with the hypersensitive response (HR), but the numbers of cells that die, the rapidity of their death, and the localization of cell death to the epidermis or its additional expression in the mesophyll, appears to be determined by the particular locus/allele responsible for the resistance (Boyd *et al.*, 1995; Kruger *et al.*, 2002). In turn, the rapidity of cell death influences the amount of growth an avirulent isolate makes before HR arrests further development. Thus, the terms 'fast-', 'intermediate-' and 'slow-acting' have been used to describe the effects of different alleles/genes (Kruger *et al.*, 2002), which can also be discriminated by their macroscopic response phenotypes (e.g. Kolster *et al.*, 1986). Death is preceded by cessation of cytoplasmic streaming followed by loss of ability to take up vital dyes or plasmolyse (Koga *et al.*, 1988). The onset of whole-cell autofluorescence is a reliable and convenient indicator of cell death (Koga *et al.*, 1988; Zeyen *et al.*, 1995; Lyngkjær *et al.*, 2001). In some cases, after the fungus establishes a functional haustorium, posthaustorial resistance may delay growth of secondary hyphae, maturation of secondary appressoria and therefore sporulation of the colony (Niks & Rubiales, 2002).

Cereal crops, including oats, show generally less powdery mildew infection on older plants than on seedlings. Greenhouse and laboratory studies have shown that this field or 'adult plant' resistance (APR) (*sensu* Robinson, 1969) is expressed by later-formed leaves of barley (Hwang & Heitefuss, 1982), oats (Jones & Hayes, 1971; Carver & Carr, 1977) and wheat (Shaner, 1973), although the magnitude differs among genotypes. This kind of resistance is highly interesting from the breeding point of view, since it appears to be distinct from 'major gene' resistance (Russell *et al.*, 1976; Hwang & Heitefuss, 1982; Wright & Heale, 1984) and hence, may be more durable. Up to seven additive genetic factors were found to contribute to this resistance in oat cv. Maldwyn (Jones, 1986). However, quantitative assessment of powdery mildew in detached leaves was not sufficiently precise to be used alone in selecting segregants for high levels of APR. Identification of the components of resistance that account for this enhancement would be highly desirable to aid breeding programmes.

The present work aimed to identify novel sources of resistance to powdery mildew caused by *B. graminis* f.sp. *avenae*, characterize the resistance responses/mechanisms at the cellular level, identify genotypes with APR and further characterize the components of this resistance. Altogether, this information would be beneficial to oat breeders in developing cultivars with durable resistance to this pathogen.

Materials and methods

Plants, pathogen and inoculation

For the screening, a germplasm collection of oat landraces containing 110 genotypes of *Avena sativa* and 31 of *Avena byzantina*, kindly provided by the Centro de Recursos Fitogenéticos, INIA, Madrid, Spain, and 24 commercial varieties supplied by the Andalusian Network of Agriculture Experimentation (RAEA) were used. Oat cv. Selma was used as the susceptible control. Seeds were germinated in Petri dishes with moistened filter papers in the dark for 48 h in a growth chamber at 65% relative humidity and 20°C. Seedlings were transplanted to 125-mL pots filled with peat:sand (3:1) and returned to the chamber. Plants were grown at 20°C, 65% relative humidity and under 12-h dark/12-h light with 200 μmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes for approximately 12 days, when plants had two expanded leaves.

Plants were inoculated with *B. graminis* f.sp. *avenae* race 5, maintained on seedlings of oat cv. Selma, in a spore-proof glasshouse. Plants were shaken 1 day before experimentation, ensuring that only young, vigorous spores were used for inoculum.

For inoculation, unless otherwise stated, when the second leaf was fully expanded (12 days), the first leaf was inoculated with *B. graminis* f.sp. *avenae* using a settling tower (Lyngkjær & Carver, 1999) to give about 30 conidia mm^{-2}. For APR assessment, 46 genotypes that previously showed low disease severity in field trials were selected. Eight plants of each of these genotypes were grown. Four out of the eight plants were inoculated on the first leaves when the second-formed leaf was expanded and the other four plants were inoculated on the fifth leaf when the sixth leaf was expanded as stated above.

Macroscopic assessment of symptom development following inoculation with *B. graminis* f.sp. *avenae*

In a first experiment, the whole collection was studied for disease severity to rule out those plants showing susceptibility from the histological studies. In this screening, four plants of each accession (Table 1) were assessed. For easier comparison among genotypes, germplasm bank codes were substituted for other codes that were easier to read. Equivalencies are given in Table 1.

After inoculation, plants were maintained in the above-mentioned growth chamber for 8 days before assessment of the percentage area covered by powdery mildew on the inoculated leaf. In the susceptible cv. Selma approximately 80% of the leaf was covered by mycelium after 8 days. Disease scores were converted into relative values, expressed as a percentage of the reading on the Selma control, and referred to as the relative disease severity (RDS) (Rubiales *et al.*, 1993; Martínez *et al.*, 2007). Thus, those extremely susceptible genotypes in which more than 80% of the leaf was covered by

Table 1 Oat landraces screened for powdery mildew resistance

Bank code	Species	Text code	Bank code	Species	Text code	Bank code	Species	Text code
BGE008113	As	Gen 1	BGE009632	As	Gen 48	BGE015355	Ab	Gen 95
BGE008115	As	Gen 2	BGE009633	As	Gen 49	BGE015356	Ab	Gen 96
BGE008116	As	Gen 3	BGE009634	Ab	Gen 50	BGE015357	As	Gen 97
BGE008117	As	Gen 4	BGE009642	As	Gen 51	BGE015358	As	Gen 98
BGE008118	As	Gen 5	BGE009643	As	Gen 52	BGE015359	Ab	Gen 99
BGE008119	As	Gen 6	BGE009644	As	Gen 53	BGE015360	Ab	Gen 100
BGE008121	As	Gen 7	BGE009645	As	Gen 54	BGE018448	As	Gen 101
BGE008122	As	Gen 8	BGE009646	As	Gen 55	BGE018452	As	Gen 102
BGE008123	As	Gen 9	BGE009647	As	Gen 56	BGE018453	As	Gen 103
BGE008124	As	Gen 10	BGE009648	As	Gen 57	BGE018454	As	Gen 104
BGE008140	Ab	Gen 11	BGE009649	As	Gen 58	BGE018455	As	Gen 105
BGE008174	As	Gen 12	BGE009668	As	Gen 59	BGE018482	As	Gen 106
BGE008175	As	Gen 13	BGE009711	As	Gen 60	BGE018483	As	Gen 107
BGE008176	As	Gen 14	BGE009721	As	Gen 61	BGE018484	As	Gen 108
BGE008177	As	Gen 15	BGE009722	As	Gen 62	BGE018485	As	Gen 109
BGE008178	As	Gen 16	BGE009723	As	Gen 63	BGE018486	As	Gen 110
BGE008179	As	Gen 17	BGE009746	As	Gen 64	BGE018487	As	Gen 111
BGE008180	As	Gen 18	BGE009754	Ab	Gen 65	BGE018488	As	Gen 112
BGE008189	As	Gen 19	BGE009748	As	Gen 66	BGE018489	As	Gen 113
BGE008200	As	Gen 20	BGE009772	Ab	Gen 67	BGE018490	As	Gen 114
BGE008201	As	Gen 21	BGE010416	Ab	Gen 68	BGE018491	As	Gen 115
BGE008202	As	Gen 22	BGE010418	As	Gen 69	BGE018492	As	Gen 116
BGE008279	As	Gen 23	BGE010419	As	Gen 70	BGE018495	As	Gen 117
BGE008382	Ab	Gen 24	BGE010420	As	Gen 71	BGE018498	As	Gen 118
BGE008397	Ab	Gen 25	BGE010421	Ab	Gen 72	BGE018499	As	Gen 119
BGE008398	Ab	Gen 26	BGE010423	As	Gen 73	BGE018501	As	Gen 120
BGE008399	As	Gen 27	BGE010424	As	Gen 74	BGE018567	As	Gen 121
BGE008400	Ab	Gen 28	BGE010425	As	Gen 75	BGE018568	As	Gen 122
BGE008401	As	Gen 29	BGE010426	Ab	Gen 76	BGE018569	As	Gen 123
BGE008402	As	Gen 30	BGE010427	As	Gen 77	BGE018570	As	Gen 124
BGE008403	As	Gen 31	BGE010428	As	Gen 78	BGE018576	Ab	Gen 125
BGE008404	As	Gen 32	BGE010429	Ab	Gen 79	BGE018577	Ab	Gen 126
BGE008405	As	Gen 33	BGE010430	Ab	Gen 80	BGE018579	Ab	Gen 127
BGE008406	As	Gen 34	BGE010431	As	Gen 81	BGE018580	Ab	Gen 128
BGE008407	As	Gen 35	BGE010432	As	Gen 82	BGE018581	Ab	Gen 129
BGE008408	As	Gen 36	BGE010433	As	Gen 83	BGE018582	Ab	Gen 130
BGE008409	As	Gen 37	BGE010434	Ab	Gen 84	BGE018583	Ab	Gen 131
BGE008477	Ab	Gen 38	BGE010498	As	Gen 85	BGE018584	Ab	Gen 132
BGE008478	As	Gen 39	BGE010499	As	Gen 86	BGE018585	Ab	Gen 133
BGE008479	As	Gen 40	BGE010500	As	Gen 87	BGE018588	Ab	Gen 134
BGE008480	As	Gen 41	BGE010501	As	Gen 88	BGE018591	Ab	Gen 135
BGE008481	As	Gen 42	BGE010502	As	Gen 89	BGE018593	As	Gen 136
BGE008679	As	Gen 43	BGE010503	As	Gen 90	BGE020322	As	Gen 137
BGE009393	As	Gen 44	BGE010504	As	Gen 91	BGE026963	As	Gen 138
BGE009394	Ab	Gen 45	BGE010505	As	Gen 92	BGE026964	As	Gen 139
BGE009395	As	Gen 46	BGE015353	As	Gen 93	BGE026965	As	Gen 140
BGE009631	As	Gen 47	BGE015354	As	Gen 94	BGE030934	As	Gen 141

As: *Avena sativa*; Ab: *Avena byzantina*.

mycelium showed more than 100% values with respect to the control Selma. Accessions with RDS values lower than 50% were retained for histological studies. The experimental design was arranged according to randomized complete blocks.

For APR assessment, leaves were inoculated and macroscopically assessed as stated above without excising the leaves from the plant. Those plants with a reduction of the infection frequency in the fifth leaf of more than 80% with respect to the first leaf were selected for histological studies.

Microscopic observations of *B. graminis* f.sp. *avenae* development and epidermal cell responses to attack

For histological studies plants were maintained in the above-mentioned growth chamber until fixation at 36, 60 and 90 h after inoculation (h.a.i.). To this end the

central 30-mm leaf segment was excised and fixed on pads moistened with 3:1 ethanol:glacial acetic acid (v/v), and cleared with lactoglycerol (equal parts lactic acid, glycerol and water), as described by Carver *et al.* (1994) to avoid displacement of ungerminated conidia and loosely attached germlings. Four plants per genotype were analysed at each fixation time.

To stain fungal structures and facilitate microscopy, a drop of aniline blue in lactoglycerol (0·1%) was placed on a coverslip and a cleared leaf segment was lowered onto the coverslip so that its inoculated surface met the stain. The coverslip was then inverted onto a microscope slide smeared with lactoglycerol to complete the mount. This procedure minimizes displacement of loosely attached germlings or ungerminated conidia (Lyngkjær & Carver, 1999). Observations were made with a Leica DM LS phase contrast microscope (Leica Microsystems) fitted with differential interference contrast and incident fluorescence attachments (blue exciter filter, max transmittance 480 nm; dichroic mirror and barrier filter transmittance >530 nm).

To assess the developmental stages at which plant resistance interfered with fungal growth, 100 germlings with mature appressoria were examined for each replicate. Outcomes of primary attacks and associated plant responses were determined on each leaf fixed 36 h.a.i. by examining 100 primary appressoria on different type A epidermal cells (adjacent to stomata) attacked by a single appressorium (Rubiales & Carver, 2000). Some epidermal cells survived attack and resisted penetration by producing an effective papilla beneath the appressorium of the fungus. Note was taken of whether germlings had penetrated successfully to form a visible haustorium within the subtending epidermal cell or a papilla had stopped the fungal development. In cases where cells were penetrated, the number of secondary hyphae and secondary appressoria developed by the germling was recorded. In some cases, penetrated epidermal cells died in response to attack following HR. In these, cell cytoplasm was disorganized, and whole-cell autofluorescence, visualized by incident fluorescence microscopy, was evident throughout the cytoplasm and cell wall (Clark *et al.*, 1995). Note was made of whether the epidermal cell and directly underlying mesophyll cells had died. On leaves fixed 60 and 90 h.a.i., many established colonies had formed secondary, hyphal appressoria and their hyphae were often entangled so that individual colonies could not be discriminated. Here, therefore, 100 randomly selected appressoria (including primary and secondary) on different type A cells were examined and the outcomes of attack and host response were recorded.

For histological evaluation of APR, in addition to the parameters previously mentioned: (i) the number of secondary hyphae, (ii) the total length of secondary hyphae, obtained by adding the lengths of each hypha, (iii) the number of digitate processes of the primary haustoria and (iv) the total length of digitate processes, obtained by adding the lengths of each individual process, were measured from 25 colonies at 60 h.a.i. To this end, pictures of each colony, including detail of the primary haustoria, were captured by a digital camera (Nikon DMX 1200F) attached to the microscope and images were analysed with ImageJ software. In addition, the number of secondary and tertiary haustoria was assessed in leaves fixed at 90 h.a.i.

For most characteristics, percentage data were calculated for each leaf replicate. For ease of understanding, means of raw percentage data are presented in tables. However, for statistical analysis, percentages were transformed to arcsine square roots (transformed value = $180/\pi \times \arcsin[\sqrt{(\%/100)}]$) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 7th Edition, after which residual plots were inspected to confirm data conformed to normality.

Results

The macroscopic assessment of the 165 *Avena* accessions showed that most of them were highly susceptible to the isolate of *B. graminis* f.sp. *avenae* used in the present study. Thus, approximately 86% of all accessions had RDS scores exceeding 60% relative to the control Selma, and a further 10% of the accessions showed moderate susceptibility (RDS scores ranging from 40% to 60% of the Selma control). Four genotypes were rated as moderately resistant (one with an RDS of 21–40% and three with an RDS <20%). All 10 genotypes with RDS scores <50% were retained for the histological studies. These included four *A. sativa*, two *A. byzantina* landraces and four *A. sativa* commercial varieties (Table 1, Fig. 1).

Microscopic assessment revealed that resistance was the result of different mechanisms that impaired fungal growth at different developmental stages and, in some

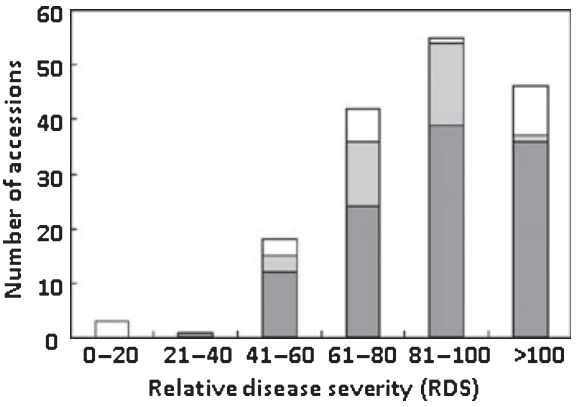

Figure 1 Macroscopic assessment of *Blumeria graminis* f.sp. *avenae* infection on *Avena sativa* (■) and *Avena byzantina* (▩) landraces and in commercial oat cultivars (□). Original disease scores were converted into relative disease severity (RDS), i.e. the percentage of the maximum reading for the cv. Selma control, 8 days after inoculation. Data are based on four plants. Accessions with <50% of the leaf covered by mycelium were selected for microscopic assessment.

cases, a combination of these mechanisms. No significant differences among genotypes were observed for conidial germination (data not shown). However, all accessions showed a significantly higher ($P < 0{\cdot}001$) level of penetration/papilla resistance compared with the susceptible control Selma (Fig. 2a). That is, epidermal cells formed an effective papilla under the site of attempted attack, preventing the penetration of the fungus. In these cases the appressorial germ tube and the appressorium formed normally but no haustorium developed inside the cell (Fig. 3a). Frequently, though not invariably, papillae in such cells showed a localized yellow/green autofluorescence that was visualized by incident fluorescence microscopy (Fig. 3b).

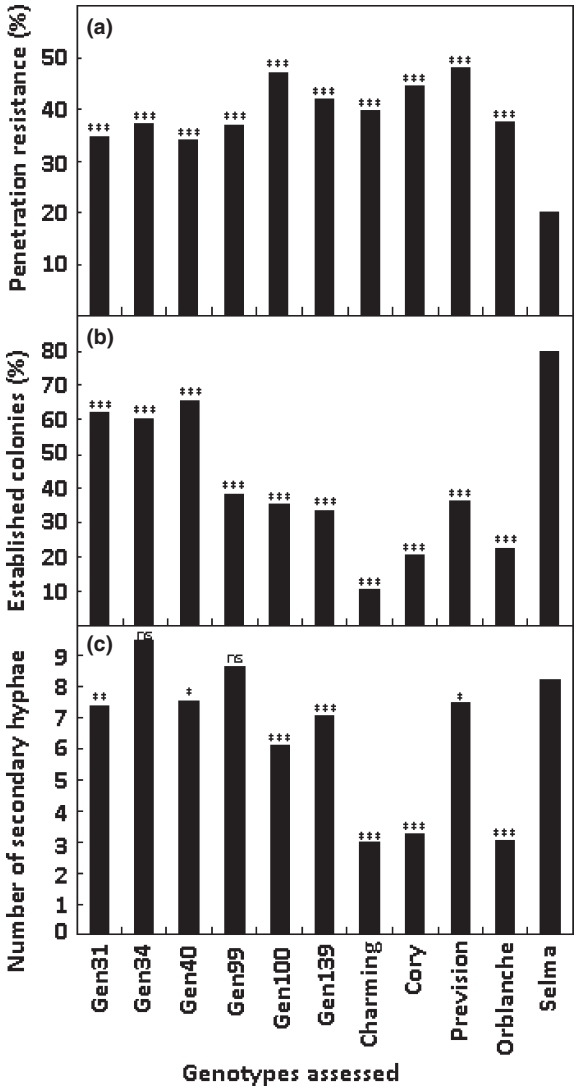

Figure 2 Microscopic assessment of *Blumeria graminis* f.sp. *avenae* infection of oat (*Avena*) genotypes 60 h after inoculation. (a) Percentage of germinated germlings that failed to penetrate the oat epidermal cell. (b) Percentage of well established colonies. (c) Number of secondary hyphae per colony. *, ** or *** above a bar indicates a significant difference between that genotype and the susceptible control Selma at $P < 0{\cdot}05$, $0{\cdot}01$ or $0{\cdot}001$, respectively; ns indicates no significant difference.

Although selected accessions showed a high percentage of penetration resistance (approximately 40% attack sites), a high percentage of papillae were overcome by the fungal penetration peg that successfully reached the cell lumen. In these cases several accessions, such as Gen99, Gen139, Charming, Cory and Orblanche, triggered the hypersensitive response that stopped further fungal development (Fig. 4). In these cases, cell cytoplasm was disorganized, and whole-cell autofluorescence was evident throughout the cytoplasm and cell wall (Fig. 5). In some accessions death was very fast and no haustorium could be observed inside the cell (Fig. 5a,b) and usually the number of dead cells increased at later fixation times (Fig. 4a). Interestingly, genotypes Charming and Orblanche also showed high percentages of slow cell death. That is, HR was not triggered immediately after cell penetration, allowing a slight development of the haustorium that was visible inside the cell (Figs. 4b & 5c,d). Also, an increase in the slow HR could be observed with time (Fig. 4b). All genotypes except Gen40 had a significantly higher percentage of total cell death than Selma. However, in Gen31 and Gen34, with percentages of cell death lower than 10%, and at late time points, this resistance response was not considered crucial for pathogen limitation (Fig. 4c). This, together with the slightly lower penetration resistance observed in these genotypes, would explain why Gen31, Gen34 and Gen 40 had higher levels of established colonies than the other resistant genotypes, even when all resistant genotypes showed significant differences from the susceptible Selma ($P < 0{\cdot}001$; Figs. 2b & 3c). In addition, genotypes Gen31, Gen34, Gen 40 and Gen 99 were also those with the highest numbers of secondary hyphae, with Gen34 and Gen99 not differing from the susceptible Selma, whereas Charming, Cory and Orblanche presented less than three secondary hyphae at time of fixation (Fig. 2c). Since the measurement of secondary hyphae was carried out in established colonies without cell death, the significant decrease in hyphal elongation was attributed at least in part to post-haustorial resistance.

Interestingly, several accessions showed high levels of resistance for different components. Thus, genotype Cory showed a high level of penetration resistance together with a high level of early HR and post-penetration resistance. In addition, Charming and Orblanche also showed high levels of slow or late HR. Altogether, the high penetration resistance, the high frequency of HR and the rapidity of its development might be responsible for the lowest macroscopic symptoms observed in these three genotypes.

According to the above data only 1·8% of the 165 genotypes screened were considered as highly resistant. Since APR had been previously reported in cereal–powdery mildew interactions, an experiment was set up in order to detect this kind of resistance in the collection for breeding purposes. Figure 6 shows an overall reduction of the disease in the fifth leaves of most of the 45

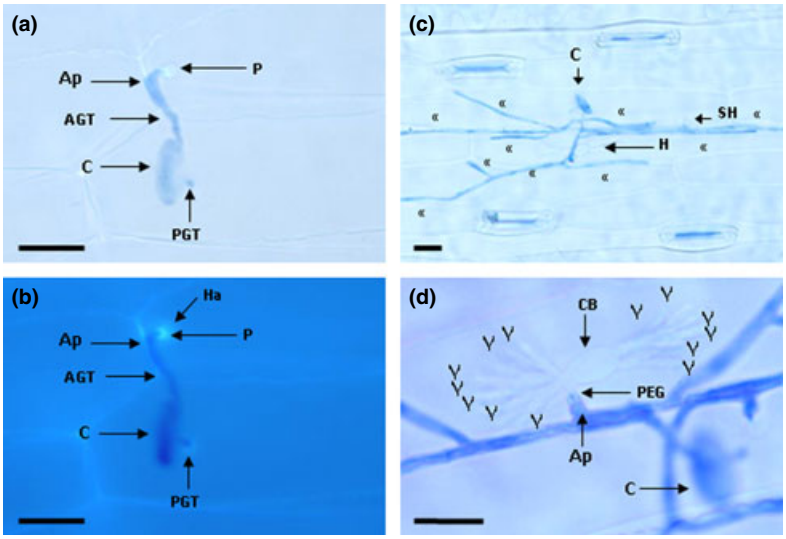

Figure 3 Transmitted light and incident fluorescence micrographs of various stages of *Blumeria graminis* f.sp. *avenae* development and plant cell responses in oat plants. Ap: appressorium; C: conidium; H: haustorium; SH: secondary haustorium; PEG: penetration peg. Bar: 25 μm in all cases. (a) The primary germ tube (PGT) has emerged from the conidium (C) and adhered to the leaf surface. The appressorial germ tube (AGT) has elongated and differentiated an apical lobe from which it attempts to penetrate the epidermal cell. (b) Blue incident light micrograph of the same cell as shown in (a); fluorescence microscopy reveals autofluorogenic material accumulated within the papilla (P) deposited as a plant epidermal cell response to the AGT. No secondary hyphae have developed, indicating penetration resistance at that site. A small papilla associated with the PGT is also evident. (c) Transmitted light micrograph of a well established colony with branching hyphae (double arrow head) and several hyphal appressoria that have developed on epidermal cells that were alive until fixation, as indicated by their failure to take up stain and the absence of whole-cell autofluorescence (not shown). (d) Detail of a haustorium which has developed several digitate processes (some indicated by single arrow heads) from each end of its central body. The penetration peg from which the haustorium swells is also evident. Ultimately, about 10 processes have formed at each end and the overall length of the mature haustorium reaches 200 μm by 120 h after inoculation.

genotypes screened, although disease reduction was highly variable. Thus, several accessions, such as Gen118, Condor, Fuwi or Selma, showed no significant differences between the first and fifth leaves for the area covered by mycelium. Several genotypes, such as Gen13, Gen126, Prevision, Acebeda, Gen18, Gen89, Gen28, Gen136 and Gen45, significantly reduced the disease by approximately 40–50% in the fifth leaves with respect to the first ones. Genotypes Gen67, Gen95, Gen47, Gen8, Gen110, Gen37, Gen50, Gen13, Gen129 and Gen128 reduced the disease by approximately 60–70%, and finally, Alcudia, Gen25, Adamo, Gen11, Gen2, Gen26, Gen38, Gen100, Gen114, Gen76 and Gen99 reduced the macroscopic symptoms by more than 80% in the fifth leaf compared with the first leaf (Fig. 6). This last set of genotypes, with the greatest reduction in symptoms, were selected for a detailed histological study in order to dissect the components of the APR observed.

Microscopic assessment revealed that most of the selected genotypes, except Gen26 and Gen100, showed significantly increased penetration resistance in the fifth leaf with respect to the first one. Overall, fifth leaves formed approximately 50% more efficient papillae than first ones, with Gen2, Gen11 and Gen25 showing the highest increases (Fig. 7a). The hypersensitive response did not add an important component to the APR since differences were not significant in most cases (Fig. 7b). Only Gen26 and Adamo significantly increased the percentage of dead cells in fifth leaves with respect to first ones, but the level did not exceed 20% total cell death. This meant that in most genotypes the reduction of the established colonies was similar to the increase in penetration resistance (Fig. 7c).

Since post-haustorial resistance has been suggested as an important component of APR, an exhaustive assessment of established colonies was carried out. Fifth-leaf colonies of all selected genotypes largely showed reduced numbers of secondary haustoria with respect to those formed in first leaves. This was also true for the susceptible cv. Selma, although the reduction was not so marked. Overall, fifth-leaf colonies had five times less secondary appressoria than first-leaf colonies (Fig. 7d). Furthermore, colonies from the fifth leaves showed a significantly lower number of secondary hyphae (Fig. 8a) and in all genotypes except Gen99 and Gen114 the total length of secondary hyphae was 1·8-fold lower in the fifth leaf (Fig. 8b). A detailed study of the primary haustoria of the colonies (Fig. 3d) showed that the number of haustorial digitate processes and their total length significantly contributed to the resistance observed (Fig. 8c,d). Thus, the number of haustorial digitate processes was 20–40% lower in colonies formed in fifth leaves than in those on first leaves. Only Gen99 and Gen100 showed no significant differences (Fig. 8c). In addition, the summed length

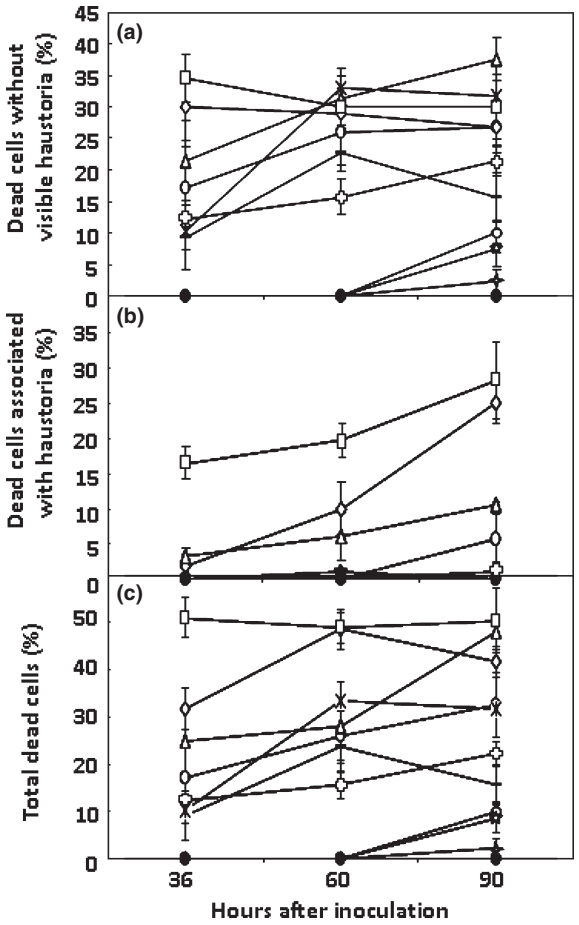

Figure 4 Assessment of cell death in oat plants attacked by *Blumeria graminis* f.sp. *avenae* at 36, 60 and 90 h after inoculation. (a) Percentage of germinated germlings associated with dead cells before haustorium was visible. (b) Percentage of dead cells associated with colonies. (c) Percentage of total dead cells. Data based on four plants. Oat genotypes: (⬡) Gen31; (☆) Gen34; (✧) Gen40; (○) Gen99; (⯃) Gen100; (✱) Gen139; (□) Charming; (△) Cory; (◇) Orblanche; (—) Prevision; (●) Selma.

of the digitate processes of each haustorium (for all genotypes) was also significantly reduced (by 36%) when formed in the fifth leaf compared to those formed in the first leaf (Fig. 8d). Altogether, data showed that, in addition to penetration resistance, all components of post-haustorial resistance greatly contributed to the APR observed.

Discussion

The purpose of this research was to find novel sources of resistance to powdery mildew and to characterize the underlying resistance mechanisms of resistant genotypes in order to aid plant breeding programmes aimed at more durable resistance. Several *A. sativa* and *A. byzantina* accessions were identified as novel sources of resistance. A range of resistance mechanisms acting at different fungal developmental stages was found in the different genotypes. This offers the possibility for combining them as different barriers to pathogen development. Interestingly, no resistance was observed at the conidial germination stage. Conidia do not normally germinate when attached to the conidiophore, although they do so rapidly upon separation. Rapid conidial germination of *B. graminis* was previously reported after suspension in a simulated airborne state (Carver & Ingerson, 1987; Carver *et al.*, 1996) or deposited on substrates with widely different characteristics (Kobayashi *et al.*, 1990; Carver *et al.*, 1996, 1999). This suggests that inhibitory processes suppress germination of attached spores and that the only stimulus necessary for germination may be separation from the conidiophore. This would explain why differences were not found among genotypes for conidial germination.

One of the most important mechanisms of basal defence is cell-wall-associated penetration resistance (Hückelhoven *et al.*, 1999; Jones & Dangl, 2006) and the most efficient penetration resistance against powdery mildew is the formation of papillae (Tosa & Shishiyama, 1984). Papillae are cell wall appositions deposited on the inner surface of epidermal cell walls directly beneath appressoria and act as physical and chemical barriers to fungal penetration (reviewed by Zeyen *et al.*, 2002). In the present study, an increase of penetration resistance was observed in the epidermal cells of all selected genotypes, Gen100 and Prevision being those with the highest penetration resistance. Phenolic compounds can be polymerized and cross-linked to form lignin or lignin-like polymers that strengthen cell walls and impede fungal penetration (Cassab, 1998). Thus, autofluorescence has often been associated with penetration resistance, although the frequency and intensity seem to differ among species and genotypes (i.e. whereas fluorescence was evident in most oat genotypes, it is difficult to discern in pea and *Medicago sativa*) (Zeyen *et al.*, 2002; Fondevilla *et al.*, 2006; Prats *et al.*, 2007). The fact that penetration resistance was frequently observed here offers opportunities for breeding for this trait. This is of high importance since papilla resistance is non-race-dependent and based on multiple and quantitative genes (Zeyen *et al.*, 2002), and therefore it is more difficult for new races of pathogens to overcome than HR.

In addition to the resistance of papillae, cell death actively contributed to resistance in several of the selected genotypes. Cell death following HR is one of the signs of an incompatible reaction between resistance (*R*) and avirulence (*avr*) gene products. Tosa & Shishiyama (1984) and Johnson *et al.* (1982) concluded that this form of resistance operates when the papilla barrier is overcome by the fungus. Therefore, accessions with high penetration resistance in addition to HR, such as Charming, Orblanche and Cory, might be highly useful as they ensure a more effective defence. In fact, it has been suggested that under field conditions HR may be relatively easily overcome by the evolving pathogen. Thus, coupling resistances affecting different phases of plant–pathogen

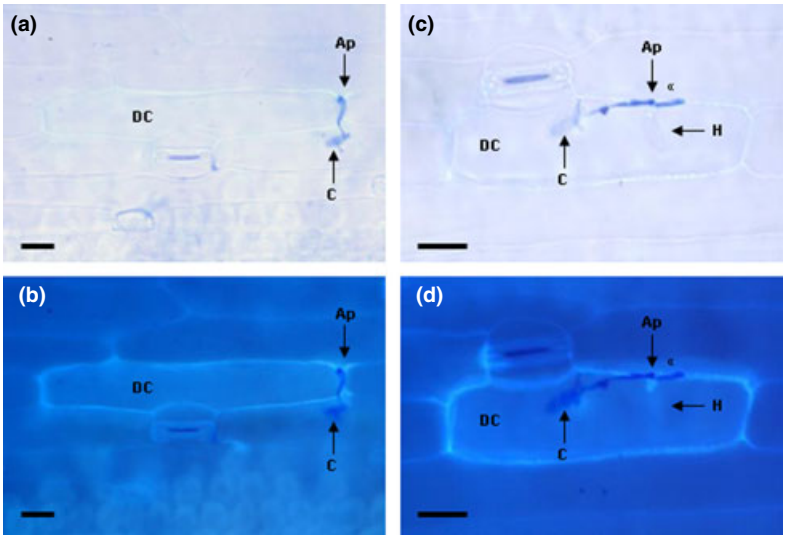

Figure 5 Transmitted light and incident fluorescence micrographs of epidermal cell death preventing further pathogen (*Blumeria graminis* f.sp. *avenae*) growth on oat plants. Ap: appressorium; C: conidium; DC: dead cell; H: haustorium. Bar: 25 μm in all cases. (a) Transmitted light micrograph. The primary germ tube (PGT) has emerged from the conidium (C) and adhered to the leaf surface. The appressorial germ tube (AGT) has elongated and differentiated an apical lobe from which it attempts to penetrate the epidermal cell. (b) Blue incident light image of the same cell as shown in (a), the epidermal cell attacked by the primary appressorium was dead at fixation as indicated by whole-cell autofluorescence. Death occurred prior to formation of a visible haustorium and no hyphae developed. (c) Transmitted light image of the fungus successfully penetrating the epidermal cell; an incipient haustorium inside the epidermal cell and a small secondary hypha (double arrow head) are evident. (d) Blue incident light image of the same cell as shown in (c), the epidermal cell attacked by the primary appressorium is dead, as indicated by whole-cell autofluorescence. The immature haustorium formed before the cell died is associated with accumulated autofluorogenic material.

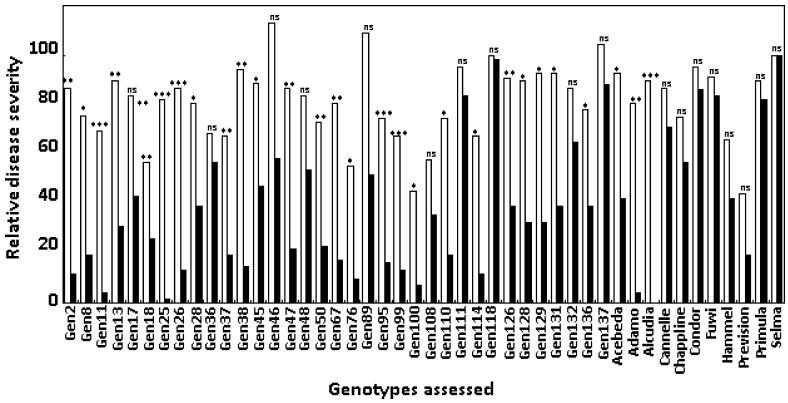

Figure 6 Macroscopic assessment of *Blumeria graminis* f.sp. *avenae* infection on first (open bars) and fifth (solid bar) leaves of 46 genotypes of *Avena sativa* 8 days after inoculation. *, ** or *** above a data pair indicates a significant difference between first and fifth leaves at $P < 0{\cdot}05$, 0·01 or 0·001, respectively; ns indicates no significant difference.

interaction and/or under genetically complex control, would present a series of barriers to pathogenesis. Such resistance should prove more durable than single-gene-controlled race-specific resistances that, although easily manipulated in plant breeding programmes, have repeatedly proved ephemeral. The detailed assessment of HR showed that not all genotypes responded in the same way. Thus, epidermal cells differed greatly in the rapidity of response in a genotype- and cell-dependent manner: some died quickly, before visible haustoria formed, while others supported functional haustoria before they eventually died, showing a 'slow' response (*sensu* Kruger *et al.*, 2002). This stochastic behaviour has also been reported in determinate barley/powdery mildew interactions (Hückelhoven & Kogel, 1998; Hückelhoven *et al.*, 1999). However, the basis of this difference and why neighbouring epidermal cells should show such contrasting response phenotypes remains unexplained. The increase of the HR with time might also be the result of the inaccessibility effect, by which the death of an

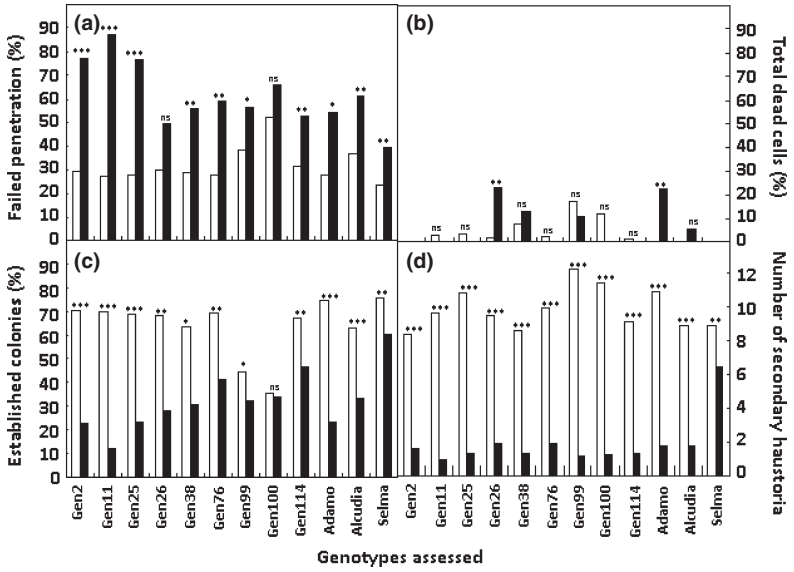

Figure 7 Microscopic assessment of *Blumeria graminis* f.sp. *avenae* infection on first (open bars) and fifth (solid bar) leaves of *Avena sativa* genotypes with adult plant resistance. Percentage of failed penetration, established colonies and dead cells were assessed at 36 h after inoculation (h.a.i.) and the number of secondary haustoria at 90 h.a.i. *, ** or *** above a data pair indicates a significant difference between first and fifth leaves at $P < 0{\cdot}05$, 0·01 or 0·001, respectively; ns indicates no significant difference.

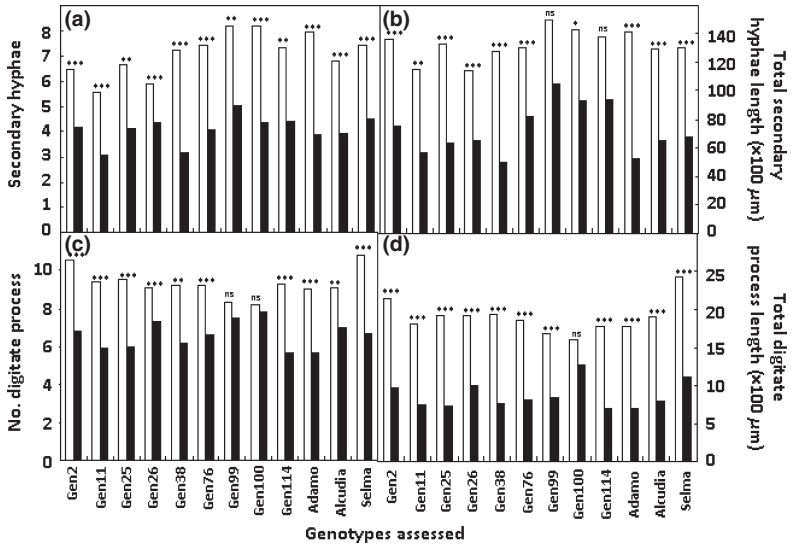

Figure 8 Microscopic assessment of *Blumeria graminis* f.sp. *avenae* posthaustorial infection on first (open bars) and fifth (solid bar) leaves of *Avena sativa* genotypes with adult plant resistance at 60 h after inoculation. *, ** and *** above a data pair indicates a significant difference between first and fifth leaves at $P < 0{\cdot}05$, 0·01 and 0·001, respectively; ns indicates no significant difference.

attacked cell predisposes the neighbouring cells to die following subsequent attacks (Lyngkjær & Carver, 1999; Prats *et al.*, 2006).

Interestingly, accessions such as Charming, Cory and Orblanche that had penetration resistance similar to the other resistant genotypes (Tukey $P < 0{\cdot}001$) showed a significantly lower number of secondary hyphae (Tukey $P < 0{\cdot}001$) than those other genotypes. One explanation for this might be the onset of a slow HR not visible as whole-cell fluorescence at the time of scoring. However, it could also be caused by mechanisms acting after haustorium formation. During establishment of a fungal biotrophic relationship the pathogen is able to control host cell processes so that defensive responses are severely impaired, while metabolic processes necessary to maintain fungal nutrition are preserved or enhanced (Leckie *et al.*, 1995). Resistance mechanisms acting after haustorium formation would lead to a restriction of nutrient

flow to the pathogen, restricting growth and delaying or impeding sporulation. However, very little is known about this quantitative post-penetration resistance and a better understanding of haustorial function in susceptibility and resistance is needed. In this sense, the detailed study of APR carried out in this work demonstrates the importance of resistance responses acting after haustorial formation in disease reduction.

Nine oat landraces and two commercial varieties with a high level of adult plant resistance were identified. Older plants of these genotypes reduced the level of infection by more than 80% with respect to the seedlings. Although APR has been described in various species, its magnitude differs among genotypes and therefore it is necessary to identify particular sources with this kind of resistance. Interestingly, seven out of the nine oat landraces corresponded to *A. byzantina* genotypes. *Avena byzantina* and *A. sativa* are the main cultivated oats and they are compatible with hybridization techniques (Stevens *et al.*, 2004), meaning they can be used in breeding programmes. Indeed, this kind of resistance is highly interesting from the breeding point of view, since it appears to be distinct from 'major gene' resistance (Wright & Heale, 1984). However, identification of the components of resistance that account for this enhancement is necessary to aid breeding. The present results show that one of the main resistance components of APR was the penetration resistance that increased in later-formed leaves of the susceptible seedlings. Furthermore, colonies developing in these later-formed leaves showed a lower number of secondary hyphae than those observed in the first leaves and haustoria did not fully develop digitate processes necessary for taking up nutrients.

It is apparent that all nutrients necessary for the elongation of secondary hyphae and colony growth must be absorbed via haustoria. Comparison of colony development on first- and fifth-formed leaves indicated that restriction of haustorial size was also an important component of APR. Restriction of haustorial development may result from several factors which include nutritional limitation or possibly physical restriction by the haustorial sheath, seen as an invagination of the host plasma membrane, separating it from the host cell (Bracker, 1968). As the sheath appears to be derived from host tissue, host genotype could exert an influence. Thus, reduction in colony size could result from a lack of host nutrients suitable for colony growth or, as the sheath seems to behave like a semipermeable membrane through which nutrients must pass before being absorbed by the haustorium (Hirata, 1967; Bushnell, 1972), sheath structure may affect nutrient passage. Altogether, the resistance described lack for the selection pressure for pathogen change implicit in that kind of resistance causing a complete block to pathogen development such as hypersensitive response. Any restriction in size and/or efficiency of haustoria must necessarily affect such characters as latent period, sporulation capacity and colony size, and so should reduce disease development. If the factors limiting colony growth can be combined with those limiting primary infection (i.e. penetration resistance), it should be possible to produce varieties with a high level of broadly based and hopefully durable resistance.

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Identification and characterization of sources of resistance in *Avena sativa*, *A. byzantina* and *A. strigosa* germplasm against a pathotype of *Puccinia coronata* f.sp. *avenae* with virulence against the *Pc94* resistance gene

J. Sánchez-Martín[a], D. Rubiales[a], J. C. Sillero[b] and E. Prats[a]*

[a]*Institute for Sustainable Agriculture, CSIC, Apdo. 4084, 14004 Córdoba; and* [b]*CIFA-IFAPA, Área de Mejora y Biotecnología, Apdo. 3092, 14004 Córdoba, Spain*

An isolate of *Puccinia coronata* f.sp. *avenae* with virulence against the oat crown rust resistance gene *Pc94* was discovered in oat fields in Córdoba, Spain. In order to identify alternative sources of resistance to this virulent isolate, a collection of 159 *Avena* accessions, including 107 *A. sativa* and 29 *A. byzantina* landraces and 23 commercial *A. sativa* and *A. strigosa* cultivars, was screened. Eight resistant landraces and four cultivars were selected according to macroscopic assessment to further characterize the operative defence mechanisms. Histological studies showed a range of defence mechanisms, acting alone or in combination, which impeded fungal development at different stages. Some accessions allowed reduced fungal growth before mesophyll penetration. In others, the fungus was arrested at the penetration stage by mesophyll cell wall strengthening and/or papilla deposition. Mesophyll cells of several accessions were penetrated by the fungus, but then the hypersensitive response (HR) leading to cell death hampered fungal development. In some cases cell death was very fast and colonies aborted early, whereas in other cases necrosis was observed later and associated with numerous secondary hyphae, suggesting a slow HR. Characterization of defence mechanisms will be useful for breeding programmes and for further cellular and molecular studies to unravel the bases of resistance. Commonalities with the resistance of the same oat collection to powdery mildew are discussed.

Keywords: hypersensitive response, oat, papilla, penetration resistance, posthaustorial resistance, rust

Introduction

Oat is a crop of Mediterranean origin used as feed grain, green or conserved fodder and, more recently, as a winter cover crop in no-till rotations (Stevens *et al.*, 2004). *Avena sativa* and *A. byzantina*, sometimes known as white oat and red oat, respectively, are the main cultivated oats. They are self-pollinating hexaploids with 42 chromosomes (6n = 42), and are compatible with hybridizing techniques (Stevens *et al.*, 2004).

Crown rust caused by *Puccinia coronata* f.sp. *avenae* is the most important disease of oat crops, causing high losses in yield and grain quality worldwide (Simons, 1985), particularly in the Mediterranean basin (Hemmami, 2006) where populations are more virulent than in the centre and north of Europe. The use of race-specific (*Pc*) genes for resistance has been the primary means of control. Presently, more than 90 genes for crown rust resistance have been assigned with permanent designations (Chong *et al.*, 2000). However these genes are unfortunately defeated rapidly by new populations of the pathogen, because of selection pressure on the pathogen resulting from large-scale and long-term cultivation practices. The gene *Pc94*, transferred from *A. strigosa*, is currently regarded as the most effective gene for resistance to *P. coronata* (Chen *et al.*, 2007), and has been incorporated into a number of oat cultivars recently released (Carson, 2008). However, virulence against this gene, albeit at low frequency, has already been detected in Canada and Europe (Jiráková & Hanzalová, 2008; Chong *et al.*, 2011). Caution should thus be taken to monitor the spread of this virulence, as it might rapidly increase as soon as *Pc94*-carrying cultivars are widely deployed.

The rust infection process starts with the germination of the urediospores on the leaf surface. When the germ tube contacts a stoma, an appressorium develops over the guard cells and then a penetration hypha penetrates through the stoma (Hoch & Staples, 1987; Prats *et al.*, 2002). The penetration hypha develops a substomatal vesicle from which a secondary hypha and a haustorium mother cell, at its tip, forms. Following contact between the haustorium mother cell and the mesophyll cell, an infection peg develops, penetrates the mesophyll cell and forms a feeding structure, the haustorium, which takes up

*E-mail: elena.prats@ias.csic.es

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nutrients for fungal growth (Parlevliet & Kievit, 1986). Attacked plants may trigger different host cell defence responses that can act before, during or after cell penetration to arrest fungal development. However, when resistance mechanisms are lacking or are insufficient to hamper fungal development, disease symptoms appear. These consist of yellow pustules containing masses of urediospores that are exposed after the rupture of the epidermis. Lesions are circular or oblong and occur on both surfaces of the leaf and can reach other green parts of the plant, when the epidemic becomes more severe. After some weeks, the borders of the pustules can turn black, with teliospore formation. When the infected plants reach maturity, production of urediospores ceases and they are then replaced by teliospores (Simons, 1985).

During the 2004–2005 and 2005–2006 growing seasons severe crown rust infection was observed on the experimental oat fields at Córdoba for the first time. The aims of the present work were to: (i) determine the virulence spectrum of this *P. coronata* f.sp. *avenae* isolate; (ii) find new sources of qualitative/quantitative resistance to this isolate in a germplasm collection of *A. sativa* and *A. byzantina* landraces and *A. sativa* and *A. strigosa* commercial cultivars; and (iii) characterize the resistance responses in order to aid breeding programmes and as a starting point for cellular and molecular studies to further investigate the bases of resistance. In addition, since this collection was also screened for powdery mildew resistance (Sánchez-Martín *et al.*, 2011), differences in the plant resistance response to the two fungi are discussed.

Materials and methods

Pathogen and plant material

Puccinia coronata f.sp. *avenae* (Pca) isolate Co-04 was derived from a bulk population collected on an oat crop at Córdoba in 2004. Urediospores were multiplied on plants of oat cvs Cory and Araceli that were highly susceptible. One day before experimental inoculation, spores were collected and kept overnight in a desiccator.

The virulence spectrum of this isolate was determined from a monosporic derivate on a set of differential genotypes possessing different resistance genes (Table 1) kindly supplied by J. Chong (Agriculture and Agri-Food, Canada) and M. Leggett (IGER, Aberystwyth, UK).

For the resistance screening a germplasm collection of landraces containing 107 genotypes of *A. sativa* and 29 of *A. byzantina* kindly provided by the Centro de Recursos Fitogenéticos, INIA, Madrid, Spain, and 23 commercial cultivars supplied by the Andalusian Network of Agriculture Experimentation (RAEA) was used. For easier comparison among genotypes and manuscript reading, germplasm bank codes were substituted with other codes easier to read (Sánchez-Martín *et al.*, 2011). The oat cultivars studied were: Ac1, Aintree, Alcudia, Araceli, Caleche, Chambord, Chappline, Charming, Cobeña, Cory, Edelprinz, Flega, Fringante, Hammel, Kankan, Kassandra, Norly, Orblanche, Pallini, Prevision, Primula, Rappidena and Saia. Oat cvs Aracely and Cory were used as susceptible controls. Seedlings were grown in 5-L trays filled with peat:sand (3:1) in a growth chamber at 20°C, 65% relative humidity and under 12-h dark/12-h light with 150 μmol m^{-2} s^{-1} photon flux density supplied by high-output white fluorescent tubes. All experiments used fully expanded first-formed leaves of 11-day-old plants (the second-formed leaf was unrolled).

Table 1 Reaction of *Puccinia coronata* f.sp. *avenae* isolate Co-04 on a set of differential oat (*Avena*) lines carrying different *Pc* resistance genes

Gene	Original source	Infection type	Reaction phenotype[a]
Pc38	*A. sterilis* CW491-4	9	S
Pc39	*A. sterilis* F-366	9	S
Pc40	*A. sterilis* F-83	9	S
Pc45	*A. sterilis* F-169	9	S
Pc46	*A. sterilis* F-290	9	S
Pc48	*A. sterilis* F-158	8	S
Pc50	*A. sterilis* Wahl No. 8	9	S
Pc51	*A. sterilis* CW-486	9	S
Pc52	*A. sterilis* Wahl No. 2	2	R
Pc54	*A. sterilis* CAV 1832	9	S
Pc56	*A. sterilis* CAV 1964	8	S
Pc58	*A. sterilis* PI 295919	7	S
Pc59	*A. sterilis* PI 296244	1	R
Pc62	*A. sterilis* CAV 4274	9	S
Pc64	*A. sterilis* CAV 4248	9	S
Pc68	*A. sterilis* CAV 4904	9	S
Pc94	*A. strigosa* RL1697	9	S
Pc96	*A. sativa*[b]	9	S
Pc1	–	9	S
Pc5	–	9	S
Pc6, *7*, *8*, *9c*, *21*	–	8	S
Pc6d	–	9	S

[a]R: resistant; S: susceptible.

[b]code unknown, line obtained from the Cereal Research Centre, Winnipeg.

Inoculation

When the first leaf was completely expanded plants were inoculated by dusting with urediospores mixed with pure talc (1:1, w/w) to give approximately 30 spores mm^{-2} (checked by counts made from glass slides laid adjacent to leaves). Homogeneous inoculation was ensured by placing the leaves attached to the plant horizontally with the help of metallic clips. After inoculation, plants were incubated for 12 h in darkness at 100% RH and 18°C, and thereafter at 20°C under a 14-h photoperiod with 150 μmol m^{-2} s^{-1} photon flux density.

Macroscopic observations

Latency period (LP), infection frequency (IF) and infection type (IT) were determined as macroscopic parameters of the disease as previously described (Prats *et al.*, 2002). LP, the time elapsing between inoculation and appearance of 50% of the pustules, was determined by

daily counting of the number of pustules visible in a 4 cm^2 marked area on the leaves. This was done by using a pocket lens (magnification × 7) until the number of pustules in the marked areas ceased to increase. The time at which 50% of the final number of pustules had appeared was estimated by interpolation. Infection frequency, given as the number of pustules per unit area, was calculated 15 days after inoculation (d.a.i.), from the same leaf area in which LP was estimated. Infection frequency scores were then converted into relative values, expressed as a percentage of the reading on the susceptible control. Infection type was determined by visual inspection of the leaf according to the 0–9 scale of McNeal *et al.* (1971).

Microscopic observations

For microscopic assessment of fungal development, middle segments of 1–3 cm^2 from each inoculated leaf still attached to the plant (four leaves per treatment) were excised 84 h after inoculation (h.a.i.) and stained with Uvitex (Ciba), according to Niks & Rubiales (1994). These were observed by ultraviolet light incident fluorescent microscopy (330 nm excitation/380 nm emission) using a Leica DM LS phase contrast microscope (Leica Microsystems; × 100 objective).

Percentages of germinated urediospores were determined from 100 random urediospores per leaf segment. Percentages of germ tubes forming an appressorium over a stoma were determined from 100 germinated urediospores per leaf segment. In addition, on each leaf segment, 100 infection units (growth arising from individual urediospore) that successfully formed an appressorium on a stoma were scored and classified according to their developmental stage, i.e. whether they formed the substomatal vesicle, penetrated the mesophyll cell and established a colony. Accordingly, early aborted infection attempts caused by penetration resistance were those that formed a substomatal vesicle and one or more primary infection hyphae, but <6 haustorial mother cells (Fig. 2c), and where colony growth had ceased. Infection units with six or more haustorial mother cells were considered established (Fig. 2f). The presence of host cell death associated with early aborted or established colonies was recorded. Death of plant cells was recognized by yellow whole-cell fluorescence under violet incident light (420 nm excitation/490 nm emission).

The length (L) and width (W) of 50 colonies per leaf segment were measured by eyepiece graticule, and colony size (CS) was calculated using the formula: $CS = \frac{1}{4}\pi LW$ (where L = maximum length and W = maximum width of a colony, in mm). Area values were transformed to square roots to obtain a linear value for colony area.

Statistical analysis

Four leaves (from four different plants) per genotype were studied in a complete randomized block design. For statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = $180/\pi \times \arcsin[\sqrt{(\%/100)}]$) to normalize data and stabilize variances throughout the data range. Data were then subjected to analysis of variance using GenStat 7th Edition, after which residual plots were inspected to confirm data conformed to normality. In addition, the Shapiro–Wilk test and Bartlett's test were performed to test normality and homogeneity of variances, respectively. Multiple comparisons among all genotypes were carried out according to the Tukey test ($P < 0{\cdot}05$).

Results

The isolate Co-04 used in this study was highly virulent, causing a compatible IT in most of the differential genotypes used in the present study, including the one carrying the resistance gene *Pc94*, and showing avirulence only on *Pc52* and *Pc59* (Table 1). According to the nomenclature proposed by Chong *et al.* (2000), the classification of this pathotype is TTNT, with virulence against all genes of subset 1 (*Pc40*, *45*, *46* and *50*), to all genes of subset 2 (*Pc38*, *39*, *48* and *68*), two genes of subset 3 (*Pc51* and *58*) and all genes of subset 4 (*Pc54*, *56*, *62* and *64*).

The macroscopic assessment of the 159 *A. sativa*, *A. byzantina* and *A. strigosa* accessions, including both the landraces and the commercial cultivars showed that most of them were moderately to highly susceptible to the isolate used in the present study. Approximately 75% of accessions had a similar or even higher infection frequency than the susceptible control (Fig. 1a). Those accessions that showed some degree of resistance with respect to the control (i.e. <80% IF) were assessed to determine their LP in order to detect accessions with mechanisms causing slow colony growth and sporulation. In most of these accessions (90%) LP was similar to the control and pustules appeared during the 7th and the 8th d.a.i. Only two genotypes, Kankan and Primula, had a LP of more than 9 days (216 h; Fig. 1b). These two genotypes, together with those considered highly resistant, resistant and moderately resistant according to their IF (i.e. ranging from 0 to 60%), were selected for histological studies in order to determine the underlying resistance mechanisms. Table 2 shows the values of the macroscopic parameters of the selected genotypes. These included eight *A. sativa* landraces and four commercial cultivars. No accessions belonging to the *A. byzantina* group fitted the selection criteria. All selected genotypes showed a high infection type with well-formed pustules, except Kankan, which showed an infection type of 1, indicating visible necrosis associated with the infection sites (Table 2).

Histological analysis of the two susceptible controls, Araceli and Cory, showed significant differences between them in several of the assessed parameters. Compared with Cory, Araceli had more established colonies without necrosis, greater colony size, more early aborted colonies without necrosis, fewer germlings not associated with a substomatal vesicle and fewer (no) early aborted colonies associated with necrosis (Table 3). Microscopy studies on the eight selected accessions and four cultivars revealed

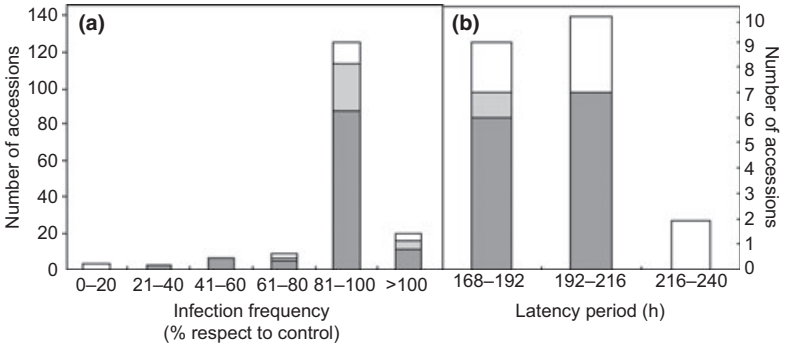

Figure 1 Macroscopic assessment of *Puccinia coronata* f.sp. *avenae* infection on *Avena sativa* (■) and *A. byzantina* (▩) landraces and in commercial oat cultivars (□) (a) Frequency distribution of infection (IF; number of pustules per cm^2 leaf) in the studied oat genotypes. Data based on four plants. Accessions from first to fourth range were selected for assessment of latency period (LP). (b) Frequency distribution of LP in the studied oat genotypes, determined by counting the number of pustules daily then grouping the genotypes in which 50% of the final number of pustules appeared during 168–192 h (7th day), 192–216 h (8th day) or after 9 days. Accessions within the first three ranges of IF and/or a LP of more than 216 h were selected for microscopic assessment.

Table 2 Macroscopic components of resistance to *Puccinia coronata* f.sp. *avenae* assessed on oat accessions selected for microscopic analysis[a]

Accession	Infection frequency[b] % relative to control	Latency period % relative to control	Infection type[b]
Araceli	100 (48·7)e[c,d]	100·0 (209·2)abc	9
Gen3	37·4 (18·3)bc	91·8 (192·1)ab	9
Gen7	59·5 (29)cd	83·9 (175·6)a	9
Gen9	60·5 (29·5)cd	90·9 (190·4)ab	9
Gen15	32·8 (16·0)abc	92·5 (193·7)ab	9
Gen18	50·3 (24·5)cd	92·2 (193·1)ab	9
Gen46	49·2 (24·0)bc	93·8 (196·4)abc	9
Gen64	51·3 (25·0)cd	96·6 (202·3)abc	9
Gen81	54·9 (26·7)cd	91·7 (192·1)ab	9
Alcudia	2·0 (1·0)a	89·8 (188·0)ab	9
Kankan	4·7 (2·3)ab	150·7 (315·5)d	1
Primula	76·9 (37·5)de	111·6 (233·6)c	9
Saia	11·8 (5·7)ab	102·7 (215·0)bc	9

[a]Those with an infection frequency similar to or <60% and/or a latency period of more than 216 h.
[b]Infection frequency and infection type were determined 15 days after inoculation.
[c]Numbers in brackets indicate raw data: pustules per square centimetre for infection frequency and hour for latency period.
[d]Different letters for each assessed parameter indicate significant differences at $P < 0{\cdot}05$ according to Tukey test.

that resistance was the result of different mechanisms/responses and in some cases a combination of them. Several accessions showed impairment of fungal development prior to mesophyll cell penetration. A low but significant reduction in spore germination was observed on leaves of Gen15, with lower values than the two susceptible genotypes, Cory and Araceli (Table 3). Rates of germ tubes successfully forming an appressorium over a stoma were in the range of *c*. 60–70%, except on accession Gen81, on which appressorium formation was significantly reduced with respect to Araceli and Cory, and cvs Alcudia, Kankan and Saia, where it was significantly reduced with respect to Cory (Table 3). This reduction in appressorium formation was caused by the germ tubes elongating without a pattern and not finding the stomata; they either passed over the stomata but did not recognize them, or formed appressoria away from the stomata. The four genotypes showed all forms of abnormality in appressorium formation without significant differences among them. About 75–90% of the sporelings that succeeded in forming an appressorium over a stoma formed a substomatal vesicle into the substomatal cavity on leaves of controls. Success in stomatal penetration and substomatal vesicle formation was significantly impaired by *c*. 50% in Gen7 and Gen64 (Table 3).

The percentage of early aborted colonies not associated with plant cell necrosis (EA-No N) was high in Gen18 (30%) and in cvs Alcudia (20%) and Saia (61%).

In several genotypes, i.e. Gen3, Gen9, Gen15, Gen46, Gen64, Gen81 and Alcudia, early aborted colonies associated with mesophyll cell death, recognized by yellow whole-cell fluorescence under violet incident light (Fig. 2d), were significantly higher than in any of the controls. Furthermore, in cv. Alcudia, in addition to the rapid cell death, a late cell death associated with established colonies could be observed (Table 3). This late cell death affected up to 44% of the infection units in Kankan and was the only microscopic resistance response observed in Primula (Table 3). In the latter, this late cell death affected several but not all of the mesophyll cells penetrated by the haustorial mother cells, so colony growth was delayed but not suppressed (Fig. 2e). This would explain the high infection frequency together with the long latency period observed in this genotype.

Interestingly several accessions showed a combination of resistance responses. The landraces Gen7 and Gen64 showed limited fungal development before mesophyll cell penetration and also a moderate level of cell death. In Saia, appressorium formation was reduced and in addition it showed a high level of penetration resistance and a moderate level of rapid cell death, lower than in Araceli.

Table 3 Microscopic assessment of *Puccinia coronata* f.sp. *avenae* development and leaf epidermal cell responses of *Avena* accessions 84 h after inoculation

Genotype	Species	Germ[a]	No App[b]	No Sub Ves[c]	EA-No N[c]	EA-N[c]	Est-N[c]	Est-No N[c]	Colony size ($mm^2 10^{-2}$)
Araceli	*A. sativa*	95·75cd	31·00abc	9·00a	7·25b	0·00a	0·00a	83·75g	8·79d
Cory	*A. sativa*	92·50bcd	21·00a	24·88bcd	0·00a	9·46b	0·00a	65·67f	6·71abc
Gen3	*A. sativa*	92·25bcd	20·75a	27·39bcd	0·48a	23·32e	0·95a	37·87d	6·61abc
Gen7	*A. sativa*	93·50bcd	28·75ab	48·87e	0·00a	20·44bcde	0·00a	30·69d	6·41abc
Gen9	*A. sativa*	90·00abc	34·25abcd	38·72de	0·00a	23·73de	0·00a	37·55d	7·38bcd
Gen15	*A. sativa*	84·00a	23·25a	31·33cde	0·00a	33·06e	0·00a	35·61d	6·25abc
Gen18	*A. sativa*	92·50bcd	27·00ab	40·39de	29·94c	12·22bcd	0·75a	16·70bc	5·90ab
Gen46	*A. sativa*	90·75abcd	23·75a	33·61cde	0·00a	27·28e	0·00a	39·11de	7·25abcd
Gen64	*A. sativa*	88·25ab	32·00abc	51·45e	0·00a	21·39cde	0·00a	27·16cd	6·56abc
Gen81	*A. sativa*	94·00bcd	50·67d	30·08cde	0·00a	36·50e	0·00a	37·00d	7·82cd
Alcudia	*A. sativa*	96·00cd	44·00bcd	17·00abc	20·33c	28·00e	28·33b	6·33a	7·38bcd
Kankan	*A. sativa*	95·25cd	47·75cd	7·25a	7·50b	11·00bc	44·00c	30·25cd	8·82d
Primula	*A. sativa*	96·33d	34·00abcd	11·33ab	8·00b	0·00a	25·67b	55·00ef	7·84cd
Saia	*A. strigosa*	93·25bcd	43·25bcd	12·25ab	61·00d	13·50bcd	0·00a	13·25ab	5·52a

[a]Percentage germination, calculated from 100 spores.
[b]Percentage of germ tubes that did not form an appressorium over a stoma, determined from 100 germinated spores.
[c]Percentage of germlings that: formed an appressorium but not the substomatal vesicle (No Sub Ves); formed the substomatal vesicle and one or more primary infection hyphae, but, because of mesophyll penetration resistance, less than six haustorial mother cells (early abortion) (EA-No N); aborted early as a result of cell death (EA-N); penetrated the mesophyll cell and established a colony (Est); established a colony but late cell death limited colony growth (Est-N); established a colony without necrosis (Est-No N); all scored from 100 infection units (growth arising from individual spore) that successfully formed an appressorium on a stoma.
Analysis of variance was applied to transformed replicate data. Different letters for each assessed parameter indicate significant differences at $P < 0{\cdot}05$ according to Tukey test.

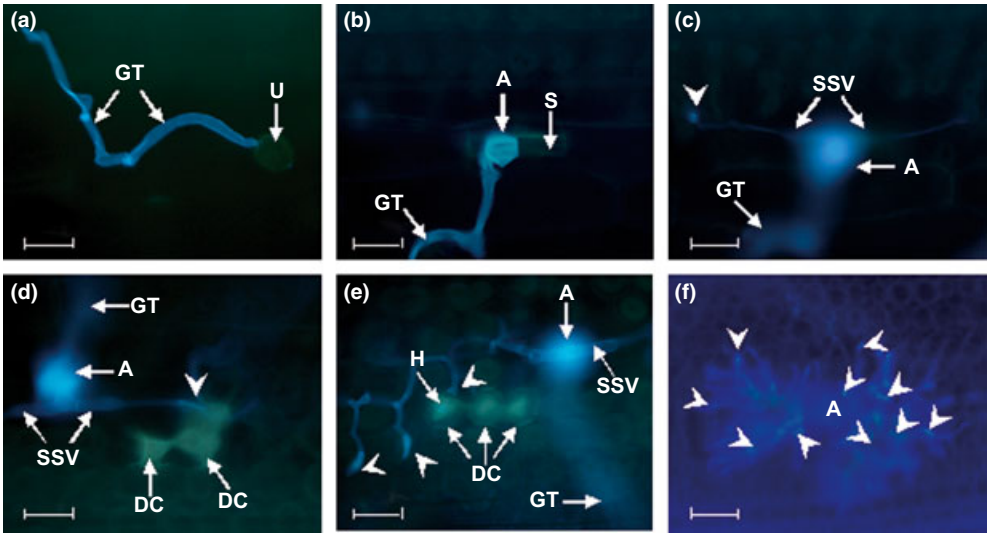

Figure 2 Light micrographs showing examples of *Puccinia coronata* f.sp. *avenae* development and leaf mesophyll cell responses of oat accessions, viewed by incident fluorescence microscopy. (a) Germinated urediospore that did not form an appressorium. (b) Urediospore germ tube forming an appressorium over a stoma but not the substomatal vesicle. (c) Early aborted colony not associated with cell death; substomatal vesicle differentiating two primary infection hyphae and a haustorial mother cell (arrow heads), but mesophyll cells are not penetrated and infection process stops. (d) Early aborted colony associated with cell death; haustorial mother cell penetrates the mesophyll cell but the penetrated and surrounding cells collapse very quickly, impeding further haustorial and hyphal development. (e) Late cell death; haustorial mother cell penetrates the mesophyll cell, which dies, but not before haustoria develop, allowing the growth of several secondary hyphae. (f) Established colony; numerous secondary hyphae not associated with necrosis indicate haustorial functionality. GT = germ tube; U = urediospore; A = appressorium; SSV = substomatal vesicle; DC = dead cell (indicated by whole-cell autofluorescence); H = haustorium; arrow heads indicate the haustorial mother cells. Bars = 25 μm.

Kankan also reduced the appressorium formation rate and showed a high level of late cell death. Finally, in Alcudia, reduced appressorium formation, a moderate level of penetration resistance and also moderate levels of early and late cell death were observed. The several resistance responses associated with these genotypes explain the low level of infection frequency observed in them (Table 2).

Discussion

This work reports virulence of *P. coronata* f.sp. *avenae* against the resistance gene *Pc94* in an isolate collected in the field at Córdoba. The pathotype isolated here was highly virulent, as shown by its classification (TTNT) according to Chong *et al.* (2000) compared to one detected in the Czeck Republic (QLBB; Jiráková & Hanzalová, 2008). It was virulent against all resistance genes of subsets 1 and 2, and to all genes of subset 4. The origin of these isolates is not known, although the simultaneous discovery of virulence in such distant areas suggests sexual recombination as opposed to spread from a nearby area. In the Mediterranean areas, where summer months are dry, the fungus may rely on sexual recombination to complete its annual cycle, thus resulting in a higher frequency of new physiological races (Reinhold & Sharp, 1982). Furthermore, several species of *Rhamnus*, the alternate *P. coronata* host, in which sexual recombination occurs, are widespread in the Andalusian area (Vadés *et al.*, 1987). The appearance of this new pathotype emphasizes the need to search for new sources of resistance, not only for race-specific resistance rapidly overcome by new fungal isolates, but also for quantitative resistance, since virulence against *Pc94* is expected to increase with the spreading of cultivars carrying the corresponding resistance gene.

Following detailed histological assessment of the resistant genotypes selected, impairment in fungal developmental stages prior to mesophyll cell penetration was observed in several genotypes. It is well known that the physical structure and chemical composition of the host surface may influence germination, germ tube elongation and appressorium formation in rust fungi (Carver & Ingerson, 1987; Gniwotta *et al.*, 2005). In addition, fungitoxic compounds constitutively excreted to the leaf surface may also interfere with germination and initial developmental stages, including the formation of substomatal vesicles (Pasechnik *et al.*, 1997; Prats *et al.*, 2007b). The observations in the present study of poor spore germination and appressoria formation support the hypothesis of mechanisms acting early during the infection process in several of the genotypes assessed. Prestomatal penetration mechanisms are frequent in non-hosts, but have seldom, if ever, been reported in hosts against their appropriate rusts (Niks & Rubiales, 2002). Notable exceptions are the low appressorium formation by various leaf rusts of cereals in some genotypes of *Hordeum chilense* (Rubiales & Niks, 1996) and of other wild barleys (Rubiales *et al.*, 1996), the reduction in appressorium formation by *Puccinia hordei* in some barley *cer*-mutants (Rubiales *et al.*, 2001) and in stomatal recognition by *Puccinia striiformis* in some resistant wheat cultivars (Broers & Lopez-Atilano, 1996).

A reduced percentage of early aborted colonies not associated with host cell necrosis, reducing the number of haustoria per colony in several accessions, particularly in Saia, was observed. Prehaustorial resistance plays a major role in so-called partial resistance (Niks & Rubiales, 2002) and has been reported in several plant/rust interactions, such as barley/*P. hordei* (Niks *et al.*, 2000), garlic/*Puccinia allii* (Fernández-Aparicio *et al.*, 2011), *Medicago truncatula*/*Uromyces striatus* (Rubiales & Moral, 2004), faba bean/*Uromyces viciae-fabae* (Sillero *et al.*, 2000), pea/*Uromyces pisi* (Barilli *et al.*, 2009), *Lathyrus sativus*/*U. pisi* (Vaz-Patto & Rubiales, 2009) or chickpea/*Uromyces ciceris-arietini* (Madrid *et al.*, 2008). An important component of prehaustorial resistance is penetration resistance, which is associated with papillae – apoplastic cell wall appositions deposited by host cells acting as physical and/or chemical barriers to attempted penetration (Zeyen *et al.*, 2002). However, reduced intercellular hyphal development has also been associated with prehaustorial resistance, as in the case of *Lr34* in wheat (Rubiales & Niks, 1995). As the current work did not study papillae formation, the mechanistic basis of this early abortion of colonies is not clear. In addition, genetic analysis would be needed to establish the inheritance of the identified resistance, as there are examples of non-hypersensitive resistance based on single genes such as *Lr34* (Rubiales & Niks, 1995) and *Lr46* (Martínez *et al.*, 2001) conferring resistance to *Puccinia triticina* in wheat, or the resistance against *U. ciceris-arietini* in chickpea (Madrid *et al.*, 2008) or against *U. pisi* in pea (Barilli *et al.*, 2010). The fact that several accessions showed prehaustorial resistance offers opportunities for breeding for this trait. This is of high importance since prehaustorial resistance is non-race-dependent and based on multiple and quantitative genes, and therefore it is more difficult to overcome by new races of pathogens than other resistance mechanisms based on single or qualitative genes, such as the hypersensitive response (Niks & Rubiales, 2002).

When penetration resistance fails and haustoria develop within host cells, another defence mechanism, i.e. a programmed cell death named the hypersensitive response (HR), can be triggered. In the present work, some of the studied accessions had a high level of HR. In several genotypes this response could be observed very early, whereas in others a slow or late cell death associated with established colonies was observed. This HR, associated with late-acting programmed cell death, has also been reported in other interactions between plants and biotrophic fungi, such as that between faba bean and *U. viciae-fabae* or between barley and *Blumeria graminis* f.sp. *hordei* (Rojas-Molina *et al.*, 2007; Prats *et al.*, 2010). According to the macroscopic assessment the combination of both early and late cell death was the most efficient response reducing IF, whereas the late cell

death observed in cv. Primula, which affected only some of the mesophyll cells penetrated by the fungus, delayed the time of sporulation but did not greatly reduce the IF. This limited effect of late-acting cell death was also observed in faba bean and barley attacked by rust and powdery mildew, respectively (Rojas-Molina *et al.*, 2007; Prats *et al.*, 2010). As the isolate used here was avirulent against *Pc52* and *Pc59*, this effect could be due to the result of the presence of either of these genes, or unidentified ones. Additional studies, such as inoculations with *Pc52*- and *Pc59*-virulent isolates and allelic tests, would be required to confirm if new genes different from *Pc52* or *Pc59* are present in the accessions displaying HR resistance.

In cv. Saia, a significant reduction of the area of established colonies was also observed. Resistance mechanisms acting after haustorium formation, which lead to a restriction of nutrient flow to the pathogen, restricting its growth and delaying or impeding sporulation, might be the cause of the reduced area of the established colonies. However, since this cultivar also showed a high level of penetration resistance, the possibility cannot be ruled out that the reduced area of the colonies was caused by this penetration resistance being not strong enough to abort the colony, but able to reduce its size.

None of the genotypes with high resistance to rust also showed high resistance to powdery mildew (Sánchez-Martín *et al.*, 2011). This could be expected for those genotypes showing a resistance response based on hypersensitivity, since this kind of resistance implies a specific gene-for-gene interaction and resistance responses greatly differ even between different isolates of the same fungal species (race-specific resistance). Interestingly, those genotypes displaying a broad-spectrum basal resistance (i.e. penetration resistance) to rust did not show the same response to powdery mildew. This might have been caused by a lack of the necessary microbe-associated molecular pattern (MAMP) receptor-mediated recognition for rust and powdery mildew, which have been reported as crucial for displaying basal defence mechanisms (Jones & Dangl, 2006).

The characterization of resistance operating in oat presented here is likely to be of high practical importance, since it can be an alternative to the *Pc94* resistance so widely used nowadays. Attempts to breed oat cultivars resistant to crown rust have been frustrated in most countries by the rapid appearance of new virulent races of *P. coronata*, often within a few years of the release of cultivars with new types of race-specific resistance. The use of plant genotypes able to stop the pathogen at different developmental stages may prove more durable and difficult to overcome by evolving pathogenic races than the use of genotypes with a single defence response or mechanisms governed monogenetically, as in the HR (Rubiales & Niks, 2000). In the oat collection studied several genotypes were found with resistance acting at several fungal developmental stages, which will allow breeding of cultivars with more durable resistance (Niks & Rubiales, 2002). On the other hand, knowledge of the mechanisms underlying resistance is important for breeding programmes since the different resistance mechanisms have different effects on plant physiology and may affect plant performance and fitness in combination with other stresses (Prats *et al.*, 2007a). There appears to be little information on the genetic background of the selected genotypes or pedigree of the selected cultivars (http://avena.agr.gc.ca). Preliminary genetic studies on the oat collection showed that the selected genotypes belong to four different clusters, indicating very different genetic backgrounds, which is interesting from the breeding point of view (E. Prats, personal communication). Selected genotypes might be crossed with the elite cultivars to increase resistance responses or used in crosses with already described resistant oats for other virulent isolates. The fact that one of the selected genotypes corresponded to the diploid black oat, *A. strigosa*, makes introgression of resistance into hexaploid *A. sativa* oats difficult due to differences in ploidy levels and of homology between chromosomes. Nevertheless, introgression is still possible and in fact, the first source of resistance containing *Pc94*, cv. Leggett, released by the AAFC Winipeg programme comes from the *A. strigosa* accession RL1607 (Carson, 2008).

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QTL's association with mechanisms of resistance to oat crown rust (*Puccinia coronata* f. sp. *avenae)* **in Ogle/TAM O-301 mapping population**

Javier Sanchez-Martin[1], Mike Bonman[2], Rubiales, D [1], Emir Islamovic[2], Adriano B. Schneider[2], Eric W. Jackson[2], and Elena Prats[1].

[1]Institute for Sustainable Agriculture, CSIC, Apdo. 4084, 14004 Cordoba, Spain.
[2]United States Department of Agriculture-Agricultural Research Service (USDA-ARS), Small Grains and Potato Germplasm Research Unit, 1691 S. 2700 W., Aberdeen, ID 83210, USA.

*To whom correspondence should be addressed:
Elena Prats
Institute for Sustainable Agriculture, CSIC, Apdo. 4084, E-14080 Córdoba, Spain.
E-mail: elena.prats@ias.csic.es
Tel: +34 957499291
Fax: +34 957499252

ABSTRACT

Crown rust caused by *Puccinia coronate* f. sp. *avenae* is the most important disease of oat crops causing yield and grain quality losses worldwide. The use of resistant varieties is one of the best alternatives to control this pathogen. The aim of this work was to characterise the resistance responses to crown rust in the population derived from the cross between the cultivated oats Ogle/TAM O-301 (OT) to further associated QTLs to the specific responses taking into advantage the genetic map developed for this population. Histological characterization showed a range of resostance responses acting both, pre and post mesophyll cell penetration. Resistance responses in the OT population were quantitatively mapped using WinQTL Cartographer. Quantitative mapping revealed QTL's originated from TAM O-301 on linkage group OT-4 and OT-19 which are linked to the hypersensitive early and late hypersensitive response (HR) observed respectively. Additionally, QTLs linked to small colony size were detected on linkage group OT-4 and OT-8 and QTL on group OT-11 was linked to resistance responses developed prior mesophyll cell penetration. QTLs linked to the specific resistance responses may prove highly useful for improving oat for a more durable resistance against rust.

INTRODUCTION

Oat crown rust is a plant disease caused by *Puccinia coronata f.sp. avenae* leading to losses in yield and grain quality worldwide (Simons, 1985). The rust infection process starts with the germination of the urediospores on the leaf surface. When the germ tube contacts a stoma, an appressorium develops over the guard cells and then a penetration hypha penetrates through the stomata (Hoch & Staples, 1987, Prats et al., 2002). The penetration hypha develops a substomatal vesicle from which a secondary hypha and a haustorium mother cell, at its tip, forms. Following contact between the haustorium mother cell and the mesophyll cell, an infection peg develops, penetrates the mesophyll cell and forms a feeding structure, the haustorium, that takes up nutrients for fungal growth (Parlevliet & Kievit, 1986). Attacked plants may trigger different host cell defense responses that can act before, during, or after cell penetration to arrest fungal development. However, when resistance mechanisms are lacking or they are insufficient to hamper fungal development, disease symptoms appear. These consist on yellow pustules containing masses of urediospores which are exposed after the rupture of the epidermis. Lesions are circular or oblong and occur in both surfaces of the foliar sheet and can reach other green parts of the plant, when the epidemic becomes more severe. After some weeks, the borders of the pustules can turn black, with teliospore formation. When the infected plants reach maturity, production of urediospores ceases and they are then replaced by teliospores (Simons, 1985).

Currently, a combination of cultural practices, such as elimination of alternate host (*Rhamnus spp*) and fungicide treatment, is diminishing the crown rust epidemics; however, no single practice is available to completely control the disease. Furthermore, the use of foliar fungicides is expensive and may threat human health and environment. Consequently, host resistance appears as the most effective, economical and environmentally friendly control method. Genetic control of oat crown rust has traditionally been performed through the use of cultivars carrying race-specific genes (*Pc* genes) involved in race-specific resistance responses. Presently, more than 90 genes for crown rust resistance have been assigned with permanent designations (Chong *et al.*, 2000). These resistance responses are useful in that they offer a complete resistance against the pathogen, however, these genes are unfortunately rapidly defeated, due to selection pressure on the pathogen as a result of large-scale and long term cultivation practices, by new populations of the pathogen. Several strategies have been adopted to improve the durability of resistance to crown rust, mainly, through the use of multilines or gene pyramiding but results are controversial. For instance, the use of multilines with Pc 51 and Pc52 genes have been shown successful in reducing rust severity in oat (Frey, 1982). whereas (Chong and Kolmer, 1993) reported the low success in pyramiding two resistance genes, *Pc38* and *Pc39*, due to the high variability of *Puccinia coronate* isolates. Thus, according to some authors these combinations of resistance genes only partially solves the crown rust problem (Carson, 2009).

In addition to race-specific resistance partial resistance or quantitative resistance have been described it oat. It is mainly based on prehaustorial responses exerted previously to mesophyll cell penetration by the germling, and due to its complex genetic control are often more durable than the race-specific resistance. Thus, resistance to peanut rust has been associated with reduced germination due to increased water repellency of the leaf surface (Cook, 1980), and failed stomata penetration has been shown to reduce disease severity in oat to the crown rust (Kochman and Brown, 1975) . Thus the characterization of the resistance responses may aid in the goal of achieving a more durable resistance. However, it could be tedious selecting those plants containing the desired resistance responses following histological evaluation of segregating populations.

The development of genetic maps via Quantitative Trait Loci (QTL) analyses has been a major breakthrough in the characterization of quantitative traits, enabling the identification of associated genomic regions and their contribution to the phenotypic variation(Fondevilla *et al.*, 2010). Thus, identification of QTLs linked to specific resistance responses might assist breeding without losing the advantage of the knowledge of the underlying resistance mechanisms.

Only a limited number of QTLs for partial resistance to crown rust have been identified and they have been determined by using the impact of the disease in agronomic traits (Diaz-Lago *et al.*, 2002) or macroscopic evaluation of disease symptoms such as disease severity and infection type (IT) (Zhu *et al.*, 2003; Acevedo *et al.*, 2010; Jackson *et al.*, 2007), hence, the specific resistance responses linked to the QTLs were not elucidated.

The Ogle/TAM 0-301 (OT) recombination inbred line (RIL) mapping population is a model population for studying crown rust in oats (Portyanko *et al.*, 2001a). Ogle, although generally susceptible in North America, were found to differ in their responses to the two crown rust isolates, PC54 and PC59 (Bush and Wise 1996). TAM O-301 also has crown rust resistance loci, *Pc58* and PcNQMG/LGCG (Hoffman *et al.*, 2006; Jackson *et al.*, 2008).

We used previously the OT linkage map developed by Portyanko et al., (2000) saturated with the available DArT markers (Tinker *et al.*, 2009) to determine QTLs linked to specific resistant responses against crown rust in oat.

MATERIALS AND METHODS

Plant material and growth conditions

The RIL population developed to construct the OT linkage map (Portyanko *et al.*, 2001a) consisting on TAM and Ogle as parents and 136 individuals derived from their crossing was increased (F_9) for this study. The population was originally developed via single seed descendent to produce F_6 seed. Individual F_6 plants were grown in the greenhouse and bulked seeds from each plant were harvested to produce $F_{6:7}$ seed. F_7 and then F_8 rows were grown to produce the $F_{6:9}$ recombinant inbred lines (RILs). In the present study, $F_{6:9}$ seed were harvested and bulked from F_8 plants which traced back to the original F_6 plants used for mapping.

Plants were grown individually in 30x110 mm plastic centrifuge tube (with two 5 mm drainage holes) filled with peat: sand (3:1) in a growth chamber with 20ºC, 65% relative humidity and under 12 h dark/12 h light under 150 $\mu molm^{-2} s^{-1}$ photon flux density.

Fungal isolate and inoculation

Puccinia coronata f.sp. *avenae* (Pca) isolate Fr-10 was obtained from Treziere (France) in 2006. Urediospores were multiplied on plants of oat cvs Cory that was highly susceptible. One day before experimental inoculation, spores were collected and kept overnight in a desiccator. The virulence spectrum of this isolate was determined from a monosporic derivate on a set of differential genotypes possessing different resistance genes kindly supplied by J. Chong (Agriculture and Agri-Food, Canada) and M. Leggett (IGER, Aberystwyth, UK).

Inoculation was performed as previously described by (Prats et al., 2007; Sanchez-Martin, 2011). Briefly, after the first leaf was completely expanded plants were inoculated by dusting with urediospores mixed with pure talc (1:1, w⁄ w) to give approximately 30 spores mm^2 (checked by counts made from glass slides laid adjacent to leaves). Homogeneous inoculation

was achieved by placing the leaves attached to the plant horizontally with the help of metallic clips. After inoculation, plants were incubated for 12 h in darkness at 100% RH and 18 ºC, and thereafter at 20 C under a 14-h photoperiod with 150 µmolm^{-2} s^{-1} photon flux density.

Macroscopic observations

Latency period (LP), infection frequency (IF) and infection type (IT) were determined as macroscopic parameters of the disease as previously described (Prats *et al.*, 2002). LP, the time elapsing between inoculation and appearance of 50% of the pustules, was determined by daily counting of the number of pustules visible in a 4 cm^2 marked area on the leaves. This was done by using a pocket lens (magnification ・x7) until the number of pustules in the marked areas ceased to increase. The time at which 50% of the final number of pustules had appeared was estimated by interpolation. Infection frequency, given as the number of pustules per unit area, was calculated 15 days after inoculation (d.a.i.), from the same leaf area in which LP was estimated. Infection frequency scores were then converted into relative values, expressed as a percentage of the reading on the susceptible control. Infection type was determined by visual inspection of the leaf according to the 0–9 scale of (McNeal *et al.*, 1971).

Microscopic observations

For microscopic assessment of fungal development, 5 replicates were prepared and microscopically assessed according to (Sanchez-Martin *et al.*, 2012) Percentage germination, calculated from 100 spores (Germ) was determinated. From 100 germinated urediospores, the percentage of germlings that did not find an stomata formed a misplaced appressorium (Misplaced appressorium) were recorded. Next, we scored from 100 infection units (growth arising from individual spore) that successfully formed an appressorium on a stoma the percentage of those that formed an appressorium over stomata but not the substomatal vesicle (OS); formed the substomatal vesicle and one or more primary infection hyphae, but, because of mesophyll penetration resistance, less than six haustorial mother cells (early abortion) (EA-No N); aborted early as a result of cell death (EA-N); penetrated the mesophyll cell and established a colony (Est); established a colony but late cell death limited colony growth (Est-N); established a colony without necrosis (Est-No N). Also, the length (L) and width (W) of 50 colonies per leaf segment were measured by eye piece reticule, and colony size (CS) was calculated using the formula: CS = ¼pLW (where L = maximum length and W = maximum width of a colony, in mm). Area values were transformed to square roots to obtain a linear value for colony area.

Quantitative mapping

Macroscopic and microscopic variability in the OT RIL population from were quantitatively mapped using WinQTL Cartographer (Wang *et al.*, 2005). QTL analysis was done using adjusted OT linkage map defined by (Jackson *et al.*, 2007) with additions of mapped crown rust resistance phenotypic markers (Jackson et al) together with new available DArT markers (Tinker *et al.*, 2009) to construct high definition OT map. Interval mapping (IM) and composite interval mapping (CIM) (Zeng, 1994) were performed for each parameter assessed. Forward regression with a threshold value of P = 0.05 was used to choose co-factors for CIM. Significant QTLs, were determined using experiment-wise significance levels established by running 1,000 permutations for all traits, $\alpha = 0.05$ (Churchill and Doerge, 1994). QTL intervals were assigned based on the area within one likelyhood of the odds (LOD) with a threshold of 2.5 QTL intervals around each QTL were established by 1 LOD minus the LOD peak approach and the percent of the phenotypic variation explained by each QTL (R^2) was calculated based on the significant marker directly to the left of the peak.

Statistical analysis

For macro and microscopic evaluations, five leaves (from five different plants) per genotype were studied in a completely randomized design. For statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = 180 ⁄ p · arcsine [(%⁄ 100)]) to normalize data and stabilize variances throughout the data range. Data were then subjected to analysis of variance using GenStat 11th Edition, after which residual plots were inspected to confirm data conformed to normality. In addition, the Shapiro–Wilk test and Bartlett's test were performed to test normality and homogeneity of variances, respectively. Significance of differences between means was determined by contrast analysis (Scheffe's). In addition least significant differences (LSD) values were added to tables and figures for comparison between each two genotypes. Association among the macro- and microscopic observations were assessed using Pearson's correlation coefficient (r) (REF). Heretability values (h^2) were estimated for each trait along de RIL population using the formula
$h^2 = \delta^2_g / (\delta^2_g + \delta^2_r)$, being δ^2_g the genetic variance, δ^2_r the error variance.

RESULTS

Macro and microscopic assessment of resistance responses

Assessment of the macroscopic disease parameters revealed that TAM 0-301 parent had an incompatible response with the tested isolate, with a IT value of 2, indicating chlorosis and necrosis that impaired the development of well formed pustules whereas Ogle showed highly susceptible (IT=9) (Table 1, Fig. 1A). Subsequent assessment of the macroscopic disease parameters in the RIL showed high IT in a high proportion of the descendent with 67% RILs showing the same IT value than the susceptible parental line Ogle and therefore in this RILs pustules were not associated to visible necrosis/chlorosis. By contrary the parental line TAM 0-301 and c.a. 35% RILs showed a low IT indicating pustules associated with necrosis/chlorosis. In addition, the resistant parental line TAM 0-301 showed a significantly longer LP that Ogle (Table 1, Fig 1A), and 10 RILs showed the same LP than the resistant parental line and 3 RIL even a longer LP (Fig 1A). Ogle parent showed also a higher infection frequency than TAM 0-301 with almost 30 fold the number of pustules per centimeter square. Interestingly, almost half of the RILs showed a similar IF than the resistant TAM 0-301 (Fig 1A). Since a high proportion of the RIL showed a high IT, the low IF suggested the presence of resistance responses not linked with necrosis in this RILs. Overall, the macroscopic assessment of the parental lines and RILs showed TAM 0-301 as highly resistant to the isolated used in this study showing macroscopically visible necrotic reaction, but also low infection frequency and latency period for which resistance responses associated to partial resistance could contribute.

To further test this possibility and to dissect the underlying mechanisms involved in the resistance of TAM 0-301 and RILs, several resistance responses that could hamper fungal infection process were assessed at microscopic level (Table 1). Histological characterization showed c.a 35% of pre-penetration resistance in TAM 0-301. Thus, 31.53% of germlings were not able to form the substomatal vesicle, either because they were not able to form an appressorium over a stomata, formed a misplaced appressorium or despite forming a appressorium over a stomata they did not formed a substomatal vesicle. Interestingly 25% of the RILs had a percentage of pre-penetration resistance similar or even higher than TAM 0-301, with a specific RIL showing up to 52% of pre-penetration resistance (Fig 1B). In addition the coefficient of variation and varianca for this resistance response was high with values of approximately 170 and 200 respectively (Table 2).

The level of penetration resistance was extremely low in Ogle, but interestingly also in TAM 0-301 (Table 1). This resistance response was not either a characteristic of the RILs with a maximun of 12,59% (Table 2). By contrast, TAM 0-301 showed high level of hypersensitive response to the tested isolate, both, early and late acting. Thus TAM 0-301 showed 12% of early aborted colonies and 44,5% of established colonies associated with necrosis (Table 1). This resistance response was also displayed in the RIL. Thus the level of early aborted colonies associated with necrosis reach 34.77% and those already established up to 76,8% (Table 2). Indeed, c.a 20% RILs showed higher level of both early and late HR than TAM 0-301. In addition the coefficient of variation and variance was very high with 76 of amplitude of the range.

The percentage of established colonies confirmed the previous data since, those parental lines and RILs characterised by a high percentage of established colonies showed a high IF and a lack of resistance responses engaged either previous or after mesophyll cell penetration (Table 1, Fig 1). Indeed, table 3 showed a significant and positive correlation between the IF and IT with percentage of established colonies, (0,66 and 0,82 respectively) whereas correlation was also high and significant but negative with the resistance responses associated with necrosis (Table 3). In line with this, high percentage of established colonies was inversely correlated with the latency period, this is, a high percentage of establishment was correlated with fast developed colonies (Table 3). Percentage of established colonies reach up to 90% in determinate RIL indicating a high susceptibility even higher than in the susceptible parent Ogle (Fig1B, Table 2).

Assessment of the colony size of established colonies not associated with necrosis showed significant differences between the parental lines. Thus, colonies in TAM 0-301 showed half the size of those developed on the Ogle leaves (Table 1). Only few RILs showed a colony size smaller than TAM 0-301, with most RIL displying values between both parental lines (Fig 1B).

QTL analysis

A total of 688 molecular markers, 309 DArT (Tinker *et al.*, 2009), 26AFLP, 5 RAPD and 348 RFLP (Portyanko *et al.*, 2001b) were assigned to 22 linkage groups. Composite interval mapping analysis identified several QTL associated with different resistance responses assessed both macro and microscopically (Table 4, 5).

One QTL marker was associated with each of the macroscopic disease parameter assessed. QTL marker CDO962A located on LG-6 was associated with IF with a LOD of 3.52 explaining 10% of the variation for this parameter (Table 4). Marker e56m11-280 located on LG-8 was associated with a LOD of 3.75 to LP explaining 12% of the variation and marker PIC21A located on LG-17 was associated with IT with a LOD of 4.18 explaining 15% of the variation (Table 4, Fig 2).

Seven additional markers were associated with the specific resistance responses histologically assessed. Interestingly the same marker, UMN853B located on LG-4, was associated with the hypersensitive response either related to early aborted or established colonies, with a LOD of 17 and 37 and explaining a 33% and 60% of the phenotypic variation respectively. An additional marker, e56m11-043 located on LG-19, was also associated with early hypersensitive response with a LOD of 8,75 and R^2 of 14%. Interestingly four markers were associated with resistance responses not related with the hypersensitve response. Thus, 2 markers, CDO708A located on LG-5 and CDO545B located on LG-11, was associated with resistance responses hampering fungal development previous to mesophyll cell penetration, explaining each of them 12% of the variation and with LOD of 3,06 and 3,86 respectively. In

addition, markers ISU54C and ISU128A located on LG-4 and 8 resoectively was associated with colony size of established colonies not associated with hyersensitive response. Marker ISU54C, with a LOD of 6,31 explained c.a. 20% of the phenotypic variation observed for this trait (Table 5, Fig, 2).

DISCUSSION

Although in this work, the parental line Ogle was susceptible to the isolated used, two major genes conferring resistance to specific *P. coronata* races have been identified in two populations, OT (Jackson *et al.*, 2007) and Kanota/Ogle (KO) (Bush and Wise, 1996). Meanwhile, TAM 0-301 has the crown rust resistance gene designated *Pc58* (Simons *et al.*, 1978) Several studies proved how *Pc58* show relative stable level of resistance over many years (Leonard, 2003; Chong and Zegeye, 2004) but, nothing about the nature of mechanism of resistance of *Pc58* is known. To date, a limited number of works have described QTLs for partial resistance to oat crown rust (Zhu *et al.*, 2003; Acevedo *et al.*, 2010; Jackson *et al.*, 2007). All of them have used macroscopic traits, like IT or DS, but nothing is known about the nature of the mechanism responsible of the resistance. Knowledge of the mechanisms underlying resistance is important for breeding programs; the combination of different mechanism can be very effective in increasing and protecting the resistance (Rubiales and Niks, 2000).

(Jackson *et al.*, 2008; Jackson *et al.*, 2010) described TAM as resistant showing IT 1 to 2, according the standard scoring system for wheat stem rust (Roelfs and Martens, 1988) and Ogle with an IT 3 to 4. From this macroscopic evaluation, we suspect that HR is involved in the resistance observed in TAM. Several components of resistance were evaluated under controlled conditions to assess the components of HR and partial resistance. The HR was evaluated through the measure of EA-N and EST-N, meanwhile EA-No-N is the best indicator of partial resistance. After the histological characterization of both parental lines, we conclude that HR is the major component of resistance in TAM O-301 and partial resistant wasn't an important component in this resistance.

Previous works have demonstrated the importance of the responses of the plant against the fungi attack in the early stages of the infection process in some rust-plant pathosystems. Study by (Prats *et al.*, 2002) demonstrated how coumarins accumulations on sunflower leaf surface prevents rust germination tube growth and appressorium differentiation, describing the "lost" stage of fungal infection. Similarly, (Rubiales *et al.*, 2001) demonstrated how barley mutants showed fewer germ tubes orientated towards stomata and were not able to recognize the stomata, describing "non-recognition" stage of fungal infection. Finally, a response by the plant against rust attack may lead that germ tubes to form an appressorium away from the stoma, describing "misplaced appressorium" stage of infection (Sillero and Rubiales, 2002; Rubiales and Moral, 2004; Patto and Rubiales, 2009).

Two QTL related with prevention of rust germination tube growth (LO) and appressorium differentiation (NR) mapped to LG-11, were purposed in the present study. Both QTL peak based on these traits, were closely associated with *P. coronata* resistance gene *Pc58a.* In a previous work (Hoffman et al 2006), three resistance genes against crown rust, designated Pc58a, b and c, were identified using six P. coronata isolates. The genetic region on OT-11 containing *Pc58a* and both QTL interval found in this study includes DArT markers oPt-11375 and oPt-6432 and the RFLP probes PSR160B.

It is well known that the physical structure and chemical composition of the host surface may influence germination, germ tube elongation and appressorium formation in rust fungi

(Carver&Ingerson, 1987; Gniwotta et al., 2005). In addition, fungitoxic compounds constitutively excreted to the leaf surface may also interfere with germination and initial developmental stages, including the formation of substomatal vesicles (Pasechnik et al., 1997; Prats et al., 2007b). Taking together the results here presented and the previous work in (Hoffman *et al.*, 2006), we could hypothesize about the implication of mechanism acting early during the infection process as responsible of resistance showed in *Pc58.* The availability of sequence information for DarT marker oPt-11375 and the RFLP probe PSR160B (NCBI) will let us do further detailed molecular genetic studies of this region.

The first major QTL identified in this study was involved in the OS and lower percentage of established colony that explained 22% of phenotypic variance. The QTL interval found in this work included the RFLP marker KSUG9, previously reported to be within the proximal region to the rust resistance gene, region ImpA (Mago *et al.*, 2002). Although there is no available information about the sequence, the fact that this molecular marker has been previously identified as involved in the rust resistance in rye and wheat, it is strongly suggested that this marker should be linked to one resistance gene or it is the resistance gene itself.

Another major QTL identified in this study was related with necrosis; neither associated with early colonies or with colonies well established that explained 52% of phenotypic variance observed. The genetic region on OT-4 containing the QTL interval found in our studies includes the probe UMN853B. This marker was reported to be placed 7.5 cM distant to a previous QTL marker for oat crown rust resistance, e35m61-050t (Jackson *et al.*, 2008). These results confirm the previous work, indicating that there is a possible gene involved to rust resistance in this genetic region. Nevertheless, the QTL identified in the previous work was related to the Uredinial length under infection, which could be involved to HR, because one of the consequences of HR response is the reduction of colony size. But future works should be designed to confirm this hypothesis.

The following QTL identified was also associated with HR, but in this case with EA-N. The genetic region on OT-4 containing the QTL peak in the marker e56m11-043, has included the marker pLRK10a, which is 2.8 cM distant of it, a putative leaf rust (*Puccinia recondita*) resistance gene clone (Feuillet et al. 1997). Another putative QTL identified in this study, that reduces the number of colonies established, is located in the genetic region on LG-4 and contain the marker e56m 12-140 as the QTL peak. Unfortunately, there is no information available from previous studies about this QTL, what is positive in one hand, since this could be a new resistance locus. On the other hand, the lack of previous information about it indicated that future studies should be developed in this genetic region with the purpose to clarify its implication to rust resistance.

The two last QTLs identified as responsible to be involved in the reduction of the colony size, with a peak on the marker ISU4C, mapped on LG-4, and the marker ISU128A, located on LG-8, were used in a previous study of comparative mapping between sorghum and maize (Pereira *et al.*, 1994). Although, no previous work related these markers to be involved with crown rust resistance. The results reported in this work suggest the importance of LG-4 in the oat crown rust, mainly the distal portion of this LG-4, where 4 QTLs involving five mechanism of resistance (OS, EA-n, Est-N, Est and CS) have represented. Additionaly QTLs have been proposed to be involved in the oat crown resistance.
Combining different mechanism of resistance in a single cultivar, theoretically, provides increased levels of resistance. However, before use this information; authors suggest the need to saturate with more markers in those genetic regions where the QTLs found. After that, the use of molecular marker linked to genes responsible for the different mechanism of resistance could allow us its use in breeding programs.

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TABLES

Table 1 Phenotypic variation among the parental lines for macro- and microscopic components of resistance to *Puccinia coronata* f.sp. *avenae*.

Parent	Macroscopic			Microscopic assessment[a]					
	IT	**IF**	**LP**	**Pre-Pen**	**EA**	**EA-N**	**Est-N**	**Est**	**CS**
Ogle	9	43.55	6.34	25.57	1.16	0	0	73.25	0.64
TAM	2	1.66^{***}	9.72^{***}	31.53^{ns}	1.29^{ns}	12.09^{**}	44.49^{***}	10.58^{***}	0.30^{**}

[a] Data obtained at 84 h.a.i. From 100 germinated urediospores, the percentage of germlings that stopped its development before mesophyll cell penetration (did not formed an appressorium over a stomata, formed a misplaced appressorium or formed an appressorium but not a substomatal vesicle, Pre-Penetration); that formed the substomatal vesicle and one or more primary infection hyphae but due to mesophyll penetration resistance form less than six haustorial mother cells (early abortion; EA); that early aborted due to cell death (EA-N); that penetrated the mesophyll cell and establish a colony (Est); that establish a colony but late cell death limited colony growth (Est-N), were recorded. Analysis of variance was applied to transformed replicate data. *, **, and *** indicate significant differences at $P < 0.05$.; 0.01 and 0.001 respectively.

Table 2 Phenotypic variation among the RILs genotypes for macro- and microscopic components of resistance to *Puccinia coronata* f.sp. *avenae*.

Parameters	Macroscopic		Microscopic assessment					
	IF	**LP**	**Pre-P**	**EA**	**EA-N**	**Est-N**	**Est**	**CS**
Ril Mean	14.45	7.57	27.26	1.09	5.78	25.38	40.34	0.576
Range	0-42.67	0-11.69	14.01-48.87	0-12.59	0-34.77	0-76.85	0-90.33	0.227-3.250
CV %	60.40	6.90	169.93	10.12	72.94	311.0	358.05	20.2
δ	832.70	7.324	201.57	10.69	264.12	2414.57	3269.91	1.6922
H	0.97	0.97	0.82	0.80	0.935	0.97	0.97	0.88

Table 3. Pearson's correlation coefficients between macroscopic and microscopic parameters scored in the RIL population derived from the cross TAM 0-301 x Ogle. Macroscopic parameters assessed were: Infection Type (IT), Infection frequency (IF), and Latency period (LP) Microscopic parameters assessed were: percentage of germlings that did not find an stomata (Lost), not able to recognize the stoma (Non Recognition; NR), that misplaced appressorium formation (Mis) and infection units in which germtubes formed an appressorium over a stoma but not a substomatal vesicle (OS), formed the substomatal vesicle and one or more primary infection hyphae but less than six haustorial mother cells (early abortion; EA); early aborted colonies due to cell death (EA-N); established colonies (Est); established colonies associated to late cell death (Est-N).

Parameter	Pre-Pen	EA	EA-N	Est-N	Est	CS
IT	0.18^{ns}	0.12^{ns}	-0.67^{**}	-0.59^{***}	0.66^{***}	0.13^{ns}
IF	0.21^{ns}	0.12^{ns}	-0.66***	-0.79^{***}	0.82^{***}	-0.14^{ns}
LP	0.03^{ns}	0.02^{ns}	0.18^{ns}	0.34^{**}	-0.35^{**}	-0.03^{ns}

Table 4: Quantitative trait locus (QTL) analysis for crown rust resistance linked to linked to macroscopic disease parameters: Infection Type (IT), Infection frequency (IF), and Latency period (LP) measured on leaves of Ogle x TAM 0-301 $F_{6:9}$ recombinant imbred lines (RIL) with *Puccinia coronate* f. sp*. avenae*

Linkage group	Trait	QTL marker[a]	QTL interval (peak)[b]	LOD[c]	R^{2d}	Additiv [e]
LG-6	**IF**	CDO962A	83.8-97.4 (84.01)	3.52	0.10	4.68
LG-8	**LP**	e56m11-280	23.9-40.4 (32.01)	3.75	0.12	0.76
LG-17	**IT**	PIC21A	85.2-104.3 (97.01)	4.18	0.15	-1.15

[a] Name of the molecular marker on the QTL peak
[b] QTL interval and QTL peak
[c] QTLs were detected using WinQTL Cartographer CIM and were based in a LOD threshold of 2.5 (1.000 permutations and a type I error of 5%).
[d] Percent of the phenotypic variation explained by the QTL.

Table 5: Quantitative trait locus (QTL) analysis for crown rust resistance linked to resistance responses impairing specific stages of the fungal infeccion process: Lost appresorial tubes (Lost), Non Recognition (NR) inability to form substomatal vesicles (OS), early aborted colonies associated with necrosis (EA-N), well established (Est), or associated with late cell death (Est-N) and Colony Size (CS) measured on leaves of Ogle x TAM 0-301 $F_{6:9}$ recombinant imbred lines (RIL) with *Puccinia coronate* f. sp*. avenae*

Linkage group	Trait	QTL marker[a]	QTL interval (peak)[b]	LOD[c]	R^{2d}	Additiv [e]
LG-4	EA-N	UMN853B	256.3-269.1 (268.01)	17.65	0.33	-4.93
LG-4	Est-N	UMN853B	256.3-269.1 (268.01)	37.94	0.60	-20.16
LG-4	CS	ISU54C	269.5-277.6 (271.6	6.31	0.19	0.16
LG-5	Pre-P	CDO708A	52.3-65.6 (59.01)	3.06	0.12	-2.27
LG-8	CS	ISU128A	143.1-149.3 (147.2)	2.90	0.08	0.24
LG-11	Pre-P	CDO545B	124.4-133.8 (128.01)	3.86	0.12	-2.28
LG-19	EA-N	e56m11-043	143.4-148.5 (147.01)	8.75	0.14	3.34

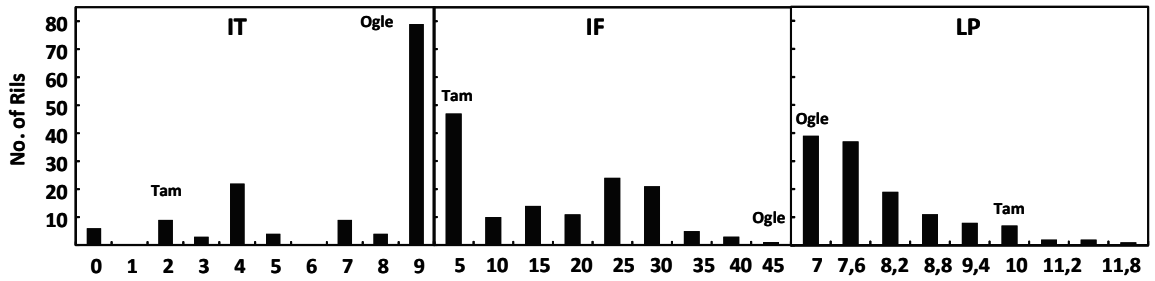
[a] Name of the molecular marker on the QTL peak
[b] QTL interval and QTL peak
[c] QTLs were detected using WinQTL Cartographer CIM and were based in a LOD threshold of 2.5 (1.000 permutations and a type I error of 5%).
[d] Percent of the phenotypic variation explained by the QTL.

FIGURE LEGENDS.

Figure 1. Histograms of the distribution of frequencies for the different disease parameters assessed. A. Macroscopic parameters**.** Infection Type (IT), Infection frequency (IF), latency Perid (LP). **B.** Microscopic parameters. Pre-penetration resistance (Pre-Penetration), Esarly aborted colonies associated with necrosis (EA-N), Early aborted colonies (EA), Established colonies associated with necrosis (Established-N), Established colonies (Established), and colony size (CS).

Figure 2. Genetic linkage groups of oat chromosomes Ogle x TAM 0-301 RIL population showing approximate positions of the markers associated with the different resistance responses. Macroscopic traits assessed were Infection Type (IT), Infection frequency (IF), and Latency period (LP). Microscopic traits assessed were early aborted colonies associated with necrosis (EA-N), well established (Est), or associated with late cell death (Est-N) and Colony Size (CS).

A


IT
IF
LP
No. of Rils
Ogle
Tam
0 1 2 3 4 5 6 7 8 9
5 10 15 20 25 30 35 40 45
7 7,6 8,2 8,8 9,4 10 11,2 11,8

B

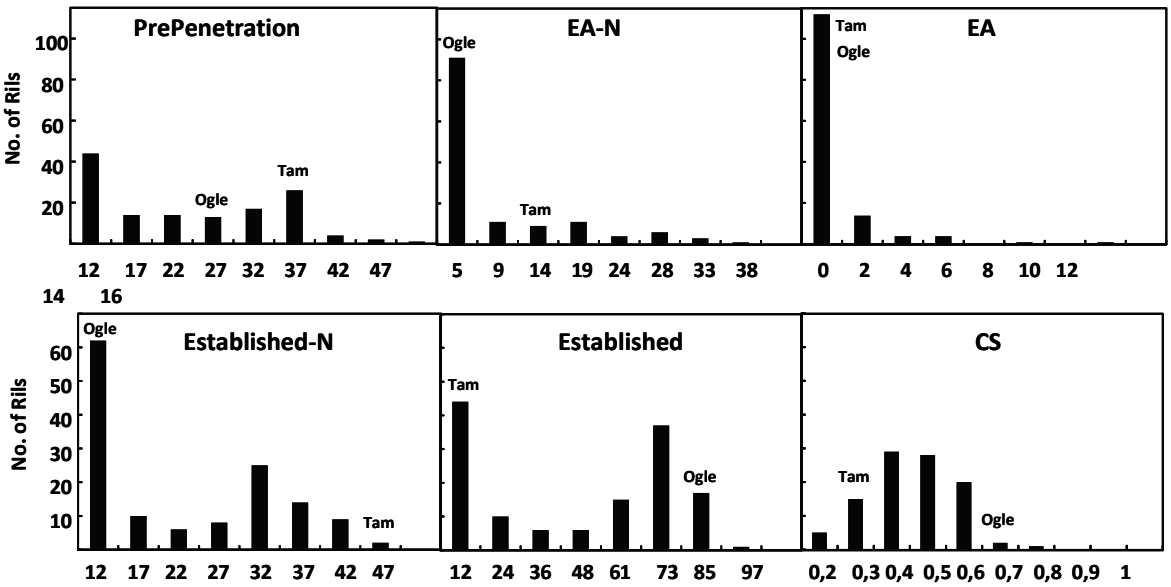

PrePenetration
EA-N
EA
Established-N
Established
CS
No. of Rils
Ogle
Tam
12 17 22 27 32 37 42 47
5 9 14 19 24 28 33 38
0 2 4 6 8 10 12
14 16
12 24 36 48 61 73 85 97
0,2 0,3 0,4 0,5 0,6 0,7 0,8 0,9 1

Figure 1.

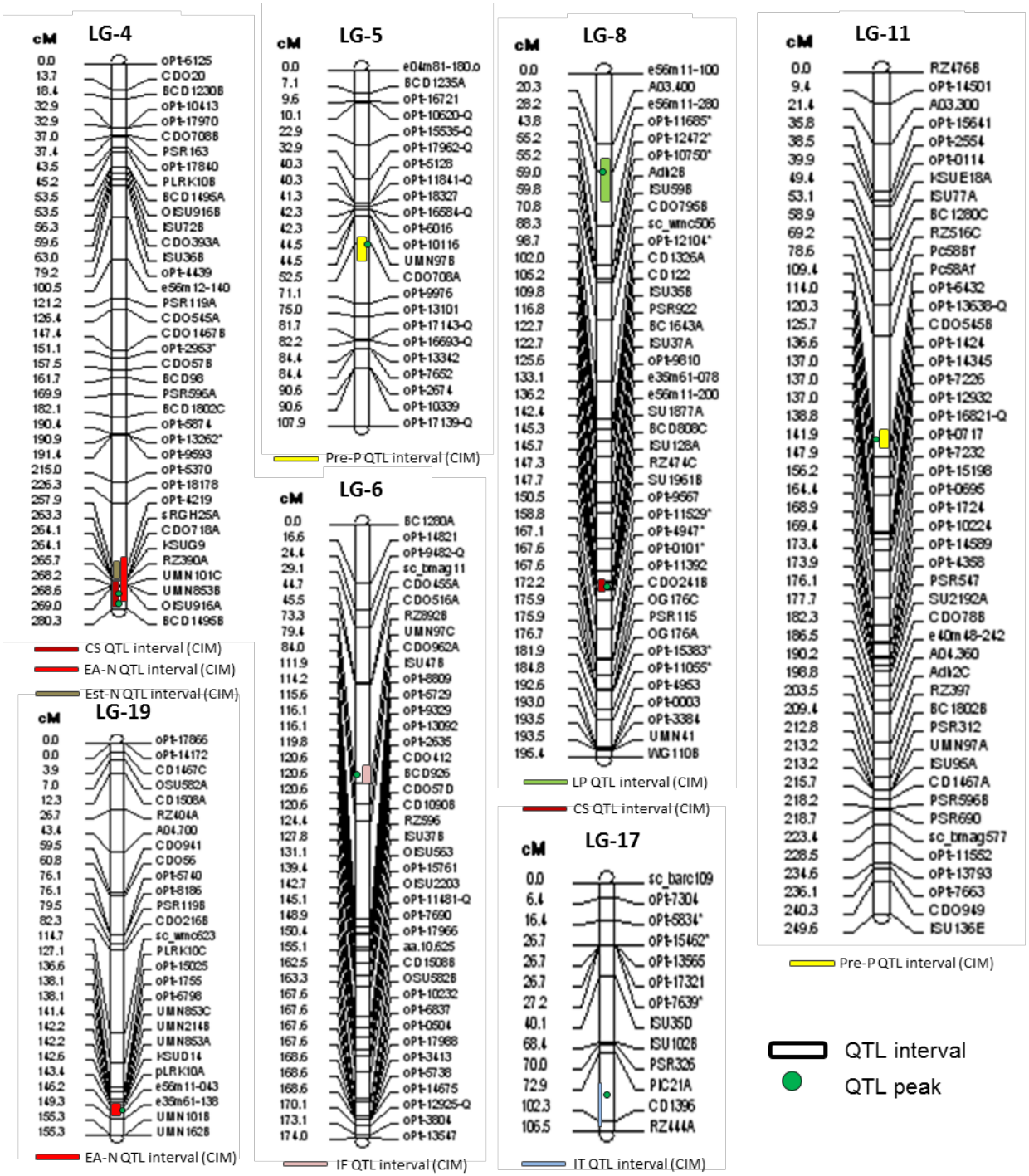

LG-4
LG-5
LG-6
LG-8
LG-11
LG-19
LG-17
cM
CS QTL interval (CIM)
EA-N QTL interval (CIM)
Est-N QTL interval (CIM)
Pre-P QTL interval (CIM)
LP QTL interval (CIM)
IF QTL interval (CIM)
IT QTL interval (CIM)
QTL interval
QTL peak

Figure 2.

Impact of hypersensitive response elicited by different life style biotrophic fungi on oat physiology under single and overlapped biotic and abiotic stresses.

Javier Sánchez-Martín[1], Luis AJ Mur[2], Diego Rubiales[1] and Elena Prats[1].*

[1]CSIC, Institute for Sustainable Agriculture, Apdo. 4084, E-14080 Córdoba, Spain.
[2]Institute of Biological, Environmental and Rural Sciences, University of Aberystwyth, UK;

*For correspondence E-mail: elena.prats@ias.csic.es
Tel: +34 957499291
Fax: +34 957 499252

ABSTRACT

The execution of resistance mechanisms against *Blumeria graminis* f. sp. *avenae* and *Puccinia coronata* f.sp. *avenae* leads to physiology alterations in oats. The main effect on plant physiology was a reduced diurnal stomatal conductance and a decrease of diverse photosynthetic parameters. Also, an increase of nocturnal stomatal conductance following HR execution was observed in the inoculated plants. There was not a direct link between the extent of the hypersensitive response and the derived physiological, stomatal or photosynthetic alterations and genetic background might in a certain manner influence the extent of the physiological dysfunctions. The pathogen life style had an important role on the physiological dysfunctions observed. The marked stomatal dysfunctions observed during oat-powdery mildew interaction suggest an important role for the proximity of dead epidermal cells in stomatal behaviour, this effect was not so accentuated following mesophyll cell death although rust appressoria formation over stomata and appressorial hyphae growing through stomatal pore into substomatal vesicle could influence the response observed following rust attack. The lack of correlation between the extent of physiological dysfunctions and percentage of dead cells and between altered photosynthetic activity and stomatal behaviour suggest an involvement of other components contributing the observed perturbations; probably ROS species produced during the execution of resistance responses. This was shown when fungal attack was overlapped with oxidative stress by high light intensity suggesting that the overproduction of ROS or the inability to cope with it might be responsible of the physiological dysfunctions.

Keywords: *Avena sativa* oat, hypersensitive response, powdery mildew, crown rust, stomatal dysfunction

INTRODUCTION

Environmental factors including biotic and abiotic stresses dramatically decrease both the crop yield and the quality of the products resulting in losses of billion euros per year. Particularly, plant pathogens are major constraints to crop growth driving large effort of the scientific community to circumvent these problems. Great progresses have already been made limiting the pathogenic pressure although results in this perpetual battle are still insufficient and were often made in detriment of environment and human health through use of toxic chemicals. The best approach to fight pathogens in a more sustainable agriculture is through the achievement of resistance cultivars. However care should be taken in the use of resistance to a particular pathogen in order to avoid undesired effects such as rapid apparition of new pathogen strains, susceptibility to other diseases or stresses, and yield penalties (Brown, 2002a; Niks and Rubiales, 2002). There has been long evidence that disease resistance may affect crop performance (Smedegaard-Petersen and Tolstrup, 1985) although most experiments were done a decade or two ago and have not been properly followed up (Brown, 2002a). This fact is important since, if resistance has a substantial cost for plants; it has a commercial significance because it may hinder the more important objective of increasing yield and quality.

The term “cost of resistance” is mainly used to explain the fact that induction of defences may be costly for the plant (Burdon and Thrall, 2003a) (Brown, 2002b; Purrington, 2000) One of the first demonstrations of the resistance cost showed that barley heavily inoculated with an avirulent isolate of *Blumeria graminis* f. sp. *hordei* (here thereafter *Bgh*) had lower grain yield, smaller grains and less grain protein than the uninoculated controls (Smedegaard-Petersen and Stolen, 1981). Nevertheless, this early evidence for the resistance cost have had remarkably little impact on thinking about disease resistance until quite recently (Brown, 2002a; Brown, 2002b; Heil, 2001; Heil and Baldwin, 2002; Heil *et al.*, 2000a). New efforts are contributing to understand the cost of disease resistance in model plans. Work in Arabidopsis has associated a cost of resistance to the presence of one *R* gene, the *Arabidopsis thaliana RPM*1 (Tian *et al.*, 2003). Authors attribute a metabolic cost to this gene, which might explain the maintenance of polymorphism for resistance and susceptibility in a plant population, although this hypothesis is still under debate (Brown, 2003; Burdon and Thrall, 2003b). However, the high cost associated to RPM1 is difficult to be explained by the metabolic cost of the gene synthesis alone, as even taken only additively the cost for all *R* Arabidopsis genes, estimated in more than 100 would rapidly become prohibitive. This paradox is recognised by the authors themselves who suggested additional factors responsible for the cost observed and related it to the induction of plant defence pathways in the absence of pathogens (Tian *et al.*, 2003). The cost necessary to induce defence and in particular the hypersensitive response has also been the explanation of the low yield increase observed in the mixtures and multilines in which individual component plants within a crop carry different *R* genes, despite the low disease level observed compared with a traditional crop. Altogether, few is known about the possible nature of the cost that has often been directly associated to the energy “lost” in inducing the defence but it was never related to the specific mechanism/s involved in the defence reaction and its effect in the plant physiology.

However our recent work in barley (Prats *et al.*, 2006; Prats *et al.*, 2010) showed that HR-mediated resistance provokes in barley stomatal dysfunctions which could be an important component of the disease resistance cost. This is important since due to the monogenic nature of this resistance mechanism it has been the most widely used in breeding programs for years. To further explore the possible implications of these HR-associated dysfunctions for crop

performance and dissect its cause work has been carried out in different oat cvs, differing in the rate of HR to two different life style pathogens, the powdery mildew and rust.

We recently showed that in barley a cost of resistance might be associated to a direct effect of the hypersensitive response on the plant physiology and hence, on the plant field performance. In fact, the HR cell death, provoked in barley by *Bgh,* lead to a paralysation of stomata that keep "lock" open with severe physiological implications even when the attacked plants appear disease free (Prats *et al.*, 2006; Prats *et al.*, 2007a). This might be therefore an important contributor of the yield penalty observed in field in plants apparently healthy, such as in the mixtures and multilines (Prats *et al.*, 2007b). In addition, plants expressing HR were unable to respond to drought stress by stomatal closure which added an additional adverse effect. This is in agreement with other authors that report a cost increase under stressful conditions (Heil, 2001; Heil and Baldwin, 2002; Heil *et al.*, 2000b).

The importance of these dysfunctions consequence of the expression of HR, one of the most popular form of resistance used by plant breeders, and the new clues that it can revealed about the cost of resistance deserve a deep consideration. One important question arisen is whether the extent of the observed dysfunctions affects similarly other species and/or all genotypes within a species. This has important implications from the breeding point of view since if different genotypes may be differentially affected by these dysfunctions, this offer opportunity for breeding. Furthermore it remains unknown whether the extent of the stomatal dysfunction is directly related to the extent of HR. Thus we analyse on oats the impact of pathogen attack in several genotypes differing in the extent of the HR induced by pathogen. A possible explanation of the stomatal dysfunction is that alteration of turgor balance of the epidermal stomatal complexes due to death of the nearby epidermal cells could cause the stomatal pore to open since opening depends on the balance between guard cell and subsidiary cell turgor. Thus, we explored the impact on plant physiology of the response elicited by two different biotrophic fungi infecting different cell types, the powdery mildew (*Blumeria graminis* f.sp. *avenae,* here thereafter *Bga*) and the crown rust (*Puccinia coronata* f.sp. *avenae, here thereafter Bca*). *Bga* is an obligate biotroph with an ectophitic habit; it penetrates only into epidermal cells to form feeding structures (haustoria) that supply the surface mycelia and reproductive structures. *Pca* is also an obligate biotroph however it enters leaves via stomata previous appressoria formation, growing into substomatal vesicles and feeding largely on mesophyll cells before rupturing epidermis at the onset of sporulation. In addition this could offer information on the generality of physiology alterations during the resistance response to different pathogens. Finally, our hypothesis is that the generation of H_2O_2 during the execution of the HR might exceed the oxidative stress with which the plant is able to cope with, hence, inducing cell dysfunctions. To test this hypothesis we superimposed a moderate-high light intensity treatment with pathogen challenge in order to determine the physiological consequences under this overlapped oxidative stress.

MATERIALS AND METHODS

Pathogen, plants, and inoculation.

Pathogens

An isolate of (*Bga)* race 5 was maintained on susceptible cultivar Selma in a spore proof growth chamber. *Bgh* CC1 was maintained on susceptible cv Pallas. Plants were shaken to remove ageing conidia 1 day before the inoculation.

(Pca) isolate Co-04, derived from a single spore collected on oat crop at Córdoba in 2004 was used. Urediospores were multiplied on plants of cvs. Araceli which were highly susceptible. One day before experimental inoculation, spores were collected and kept overnight in a desiccator.

Plant material

Three oat (*A. sativa)* cultivars, Cory, Charming and Orblanche, previously identified as resistant to *Bga* race 5 and one susceptible (Selma) (Sánchez-Martín et al., 2011), were used for study of the oat-powdery mildew interaction. In addition the barley genotype P01 (Kolster et al, 1986) previously used in the characterisation of the stomatal dysfunction in barley was added for comparison. Three oat (*A. sativa)* cultivars, Alcudia Kankan and Primula, and one (*A. strigosa)* cv, Saia, identified previously as resistant (Sánchez-Martín et al., 2012) and one susceptible (Araceli) were used for study of the oat-rust interaction.

Plants were grown individually in 30x110 mm plastic centrifuge tube (with two 5 mm drainage holes) filled with peat:sand (3:1). Tubes stood in trays filled to ca 50 mm depth with the same compost which was watered freely throughout. Plants were grown in a room with 20ºC, 65% relative humidity and under 12 h dark/12 h light. Two different light regimes were applied to plants. Half of plants grew under a non stressed light intensity regimen of 150 µmol m-2 sec-1 photon flux density (here thereafter low intensity, LI) and the other half grew under a moderate light intensity regimen of 450 µmol m-2 sec-1 photon flux density supplied by high-output white fluorescent tubes (here thereafter high intensity, HI).

Inoculation

For powdery mildew inoculation when the second leaf was fully expanded (12 days), the first leaf was inoculated with *B. graminis* f.sp. *avenae(Bga)* using a settling tower (Lyngkjær & Carver, 1999) to give about 30 conidia mm^{-2} (checked by counts made from glass slides laid adjacent to leaves). For rust inoculation, first leaves were inoculated with urediospores mixed with pure talcum (1:1, w/w) by dusting them over the plants by using a settling tower to give approximately 30 spores mm^{-2}. After inoculation, plants were incubated for 9.5 hours in darkness at 100 % RH and 18^{o}C, and thereafter at 20 ^{o}C under a 14 h photoperiod with 150 µmol m^{-2} sec^{-1} photon flux density.

Microscopy

For histological studies of powdery mildew inoculated leaves, plants were maintained in the above mentioned growth chamber until fixation at 36 h.a.i. Then the central 30mm leaf segment was excised and fixed on pads moistened with 3:1 ethanol:glacial acetic acid (v/v), and cleared with lactoglycerol (equal parts lactic acid, glycerol and water), as described before (Carver et al., 1994) to avoid displacement of ungerminated conidia and loosely attached germlings. Four plants per genotype were analyzed at each fixation time under white and ultraviolet light incident fluorescent microscopy (330 nm excitation/380 nm emission) using a Leica DM LS phase contrast microscope (Leica Microsystems, Wetzlar, Germany; 40x objective).

For microscopic assessment of rust development, central 30mm leaf segments from each inoculated leaf still attached to the plant (four leaves per treatment) were excised at 84 hours after inoculation (h.a.i.) and stained with Uvitex (Ciba, Barcelona, Spain), according to Niks and Rubiales (Niks & Rubiales, 1994). These were observed by ultraviolet light incident fluorescent microscopy (330 nm excitation/380 nm emission) using a Leica DM LS phase contrast microscope (Leica Microsystems, Wetzlar, Germany; 63x objective).

Percentages of germlings hampered in the infection process before or at time of mesophyll cell penetration (Pre-Penetration resistance), percentage of germlings inducing early or late cell death and percentage of established colonies were determined from 100 germinated urediospores per leaf segment (early or late, Sánchez Martín et al.,2011; 2012).

Stomatal conductance

Leaf water conductance (g_l) was measured in ten plants per genotype with an AP4 cycling porometer (Delta-T Devices Ltd, Cambridge, UK) according to Prats et al., (2006). g_l is the sum of epidermal and stomatal conductance, but as epidermal conductance of oat is low, changes in g_l largely reflect changes in stomatal aperture. The porometer allows rapid measurement that is non-destructive and samples a relatively large area (17.5 x 2.5 mm) of leaf. It was used on the centre of the adaxial surface of leaf laminae. Measurements were carried out in the second leaves twice a day, three hours after the onset of the light period and two hours before the end of the dark period. In light, a single measurement took <20 s and 10 replicate plants were measured in under 5 min. In darkness it took slightly longer because g_l was lower. In each experiment, sets of 10 healthy and 10 inoculated plants of the chosen genotypes were measured, each set being held in adjacent trays on the growth room bench. The porometer was wiped clean after measuring inoculated leaves to avoid transferring the pathogen. Measurements in the powdery mildew highly susceptible cultivar "Selma" were stopped earlier than in the other cvs when sporulation appeared in order to avoid experimental interferences.

Chlorophyll fluorescence analysis

Fluorescence quenching analysis were measured using modulated fluorescence on second leaves of dark-adapted plants with a PAM 2100 Fluorometer (PAM-2000; Walz, Effeltrich, Germany). Measurments were made in four different replications according to (Lichtenthaler *et al.*, 2005)

The initial fluorescence level (Fo) was determined after dark adaptation (at least 30 min) with a pulsed low red measuring light (ML) (0.1μmol photons m^{-2} s^{-1}). Then, a 1-s saturating light pulse (aprox. 6.000 μmol photons m^{-2} s^{-1}] of white light was applied to measure the maximal fluorescence (Fm) value. When fluorescence returned again to the F_o level, plants were illuminated by a non-saturating continuous red "actinic light" (AL) (655nm) for 5 minutes to drive photosynthesis and gives F' values. During the AL induced fluorescence kinetics, saturating pulses were applied with 20-s intervals in order to keep track of the fluorescence parameters Fm'(the maximum fluorescence level in the light-adapted state). After several minutes under AL, and when the F value remained low and constant, AL was turned off and a far-red radiation (735 nm) that excited preferentially photosystem (PS) I was switched on during 4 seconds to determine the value of Fo´. During the induction kinetic induced by AL the ML is automatically switched to a higher frequency of 100kHz in order to achieve a better signal to noise ratio and time resolution. In ML, while measuring F_0, the frequency of 100kHz is replaced by 1.6kHz to avoid any chlorophyll fluorescence induction kinetics. Based on these basic parameters obtained, other parameters with significant physiological relevance such as i) Maximum Quantum Yield of PSII photochemistry (F_v/F_m) and ii) PSII operating efficiency (F_q'/F_m' also termed $\Delta F/F_m$`) were derived.

Dark relaxation measurements

Dark relaxation kinetics were used to determinate the photoinhibitory quench (q_I) caused by photoinhibiton of PSII units, a major constituent of the non-photochemical quench. Following turning off of the AL during the induction kinetics, a saturation pulse was applied within the first minute of darkness and then at 5 and 20 minutes. ML remained switched on throughout the dark relaxation measurements of q_N. Then Fm`1, Fm`5 and Fm` 20 were obtained and used to determinate $_N$Fvalues (= Fm-Fm´) and hence q_N ($_NF/F_v$). The photoinhibitory quench, $q_{I,}$ was calculated as $_NF20/F_{v.}$ (Lichtenthaler 2005).

Statistical analysis

Four leaves (from four different plants) per genotype were studied in completely randomized designs. For ease of understanding, means of raw percentage data are presented in tables and figures. However, for statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = 180/π x arcsin[√(%/100)]) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 7th Edition, after which residual plots were inspected to confirm data conformed to normality. Significance of differences between means was determined by contrast analysis (Scheffe's). In addition least significant difference (LSD) values were added to tables for comparison.

RESULTS

Microscopic response of oat cultivars to Bga and Pca

Previous reports have shown in barley a correlation between the execution of the HR and stomatal lock-up leading to physiological dysfunctions. In other to test the generality of the effect and to determine whether the extent of the HR could be related to the extent of stomatal lock-up several oat cultivars differing in the percentage and the timing of HR development following *Bga* or *Pca* inoculation were evaluated.

As expected cv. Selma showed highly susceptible to *Bga* with more of 78% colonies established and no sign of HR. A slightly low 21.5% showed some levels of penetration resistance due to papilla formation (Table 1). Cory and Charming showed similar levels of HR, near to 50%, and also similar to the barley control P01. However, whereas in Cory most of the HR developed very fast before any haustorium could be seen in the epidermal cells, Charming were characterised by a high proportion of late cell death. This two oat cultivars also showed similar and high levels of penetration resistance, c.a. 45%, also similar to those observed in the barley P01. By contrary cv. Orblanche showed slightly low levels of HR both, early and late, compared with Cory or Charming and also lower level of penetration resistance. All oat cvs. were significantly more resistant than the susceptible Selma to *Bgh* (Table 1).

On the other hand cv. Araceli showed susceptibility to *Pca* with approximately 60% colonies established and no signs of cell death (Table 1). Alcudia and Kankan showed high levels of HR with approximately 30% of attempt penetration leading to cell death. However, whereas in Alcudia HR was both early and late, in Kankan the response was a late response allowing haustoria to growth for a certain time, albeit, colonies did not reach establishment. Primula with approximately 15% showed half the levels of HR observed in Alcudia and Kankan. Finally we also studied cv. Saia highly resistant to *Pca* but not based in cell death mechanisms but in penetration resistance. All oat cvs. were significantly more resistant than the susceptible Araceli to *Pca* (Table 1).

Physiological response of HR-based oat cvs. to Bga challenge

Stomatal conductance

Under low, non-stressing light intensity, stomatal conductance during the day ranged by mean between 300 and 500 µmol $m^{-2}s^{-1}$ in control, healthy oat plants. As expected following inoculation a significant reduction on stomatal conductance ($p<0.001$) was observed in barley P01 confirming previous results. However, this trend was only observed in the oat cv. Cory that showed a reduction of approximately 15% on the AUDPC values on inoculated plants respect to the control ($p<0.001$, Fig. 1). The overall reduction in Cory was similar to that observed in P01. Interestingly during the night most cvs showed increased conductance following powdery mildew inoculation. Thus, Cory, and Orblanche showed increased stomatal conductance from 35 h.a.i ($p< 0.001$) with overall increases of 31.74% and 82.72% respectively in the AUDP curves. These increases were nevertheless far from the 141.9% increase in stomatal conductance observed in the barley P01 ($p<0.001$, Fig. 2). Charming showed no significant differences related to stomatal conductance between control and inoculated plants during the complete time course of 185 hours (Fig 2). The inoculated susceptible Selma plants also showed overall a higher AUDPC values than the healthy plants ($p=0.05$, Fig 2) although the pattern was slightly different from the resistant cvs since the increase in Selma was only significant from 109 h.a.i. (Fig 2). Measurements on Selma were stopped from this time point since its sporulation could favour interferences with the other cvs.

Photosynthetic parameters

Challenge of resistant oat cvs with *Bga,* clearly affected the photosynthetic parameters of the plants grown under low, non-stressing light intensity. Control plants showed a maximum quantum efficiency (Fv/Fm) of approximately 0.75 for all genotypes. However, *Bga* inoculation reduced Fv/Fm ratio significantly in all cvs (Fig. 3).The higher reduction in Fv/Fm were observed in cv Cory with reduction up to 0.10 units whereas Charming and Orblanche showed slightly lower reduction. The highest reduction was observed in the barley P01 with reduction up to 0.20 units. Interestingly the highest reductions were observed from 7-8 days after inoculation, depending on the cvs (Fig. 3).

The potential dysfunction affecting photosynthetic machinery was also observed following assessment of the operating efficiency of photosystem II (PSII). Whereas control, healthy plants showed levels near to 2.00. Following *Bga* inoculation these values decrease in most cvs. Cory showed the highest reduction in the PSII operating efficiency with values similar to that observed in the barley P01. Charming significantly reduced its operating efficiency ($p=0.029$) although not to the extent observed in Cory. PSII operating efficiency of Orblanche was the unique not significantly affected by *Bga* inoculation (Fig. 4). To further study the possible damage of the photosynthetic system reflected by the reduction in the maximum quantum and operating efficiency, the level of photoinhibition following dark relaxation measurements were recorded. Photoinhibition increased significantly in Cory ($p=0.013$) and in the barley control P01 ($p=0.004$) although the increase observed in P01 was far more pronounced than that observed in the oat cvs (Fig. 4).

Physiological response of HR-based oat cvs. to Pca challenge

Stomatal conductance

Similar to that observed following *Bga* inoculation*,* following rust inoculation under low, non-stressing light intensity, diurnal stomatal conductance was reduced significantly in most resistant oat cvs. Primula and Saia significantly reduced its stomatal conductance from 62 h.a.i. ($p=0.023$ and $p<0.001$ respectively), whereas Alcudia showed a significant reduction from

108 h.a.i ($p<0.001$), (Fig. 5). Overall AUDPC values decreased also significantly with a reduction of approximately 19% in, Primula, Saia and Alcudia (Fig 5). Inoculated leaves of the resistant cv. Kankan showed no difference relatives to the diurnal stomatal conductance with respect to healthy controls plants (Fig 5). Susceptible cv. Araceli also reduced its diurnal conductance consequence of the *Pca* inoculation. Interestingly and unlike the effect observed following *Bga* challenge, in general nocturnal stomatal conductance was not highly affected by rust inoculation. Only in Alcudia and Saia rust inoculation significantly affected nocturnal conductance ($p=0.018$ and $p=0.025$ respectively) albeit the effect was the opposite to that observed following powdery mildew inoculation, this is, reducing instead of increasing conductance (Fig. 6).

Photosynthetic parameters

As previously observed maximum quantum efficiency of oat cvs were approximately of 0.75 (Fig 7). Following inoculation, Alcudia and Saia significantly reduced Fv/Fm values ($p<0.001$) from the first day after inoculation and this reduction was also evident in Kankan and Primula from day 5 after inoculation (Fig. 7). However these differences were not so pronounced as those observed following powdery mildew inoculation with changes of around 2 units after rust attack. Similarly, only few changes in PSII operating efficiency were observed following rust inoculation. A slightly significant decrease in this photosynthetic parameter was observed in challenged Primula leaves compared with healthy ones (Fig. 8) whereas no significant differences were observed in the others cvs. In addition a significantly increase of photoinhibition was detected in Saia ($p=0.004$) after rust inoculation (Fig 8).

Physiological response of oat cvs. under overlapped biotic and abiotic stresses

Stomatal conductance

Plants grown under high (moderately stressing) light intensity conditions (HL) showed a very different stomatal conductance pattern, both diurnal and nocturnal, with respect to the non-stressed low light intensity (LL) grown plants. Thus, most oat cvs. such as Alcudia, Araceli, Kankan or Saia and the barley P01 significantly reduced the stomatal conductance under HL with reductions ranging from 11.01% in Kankan to 62.96% in Alcudia. Others oat cvs such as Primula, Charming and Orblanche did not change the diurnal conductance respect to healthy LL growing plants and Cory significantly ($p<0.001$) increased its conductance when growth under HL conditions.

Similarly to the effect observed under LL conditions, following powdery mildew inoculation of the HL adapted plants, the resistant cv Cory, the barley P01 and in addition oat cv Orblanche, which previously were not affected, significantly reduced their diurnal conductance (Fig 1). However the most interesting effect was observed after nocturnal conductance assessment. Similar to the effect provoked by powdery mildew, HL treatment induced a significant increase of stomatal conductance in non-inoculated leaves although in this case differences were significant for all resistant cvs ($p<0.001$). This increase was of 50.26% in Cory or 53.4% in P01, so it was slightly higher than the increase induced by powdery mildew challenge in Cory but much lower in P01. Interestingly when overlapped powdery mildew inoculation with the HL treatment, oat nocturnal conductance dramatically increased during the first 60 h.a.i. to maintain thereafter a steady state of highly elevated conductance (Fig. 2). The barley P01 showed a continuous increase over the whole period assessed reaching by the last measurements similar values to that measured during the day indicating a complete lock-up. The overlapped *Bga* challenge with the HL increased significantly the already high conductance observed under the HL conditions in more than 2 fold in all genotypes, with the

higher increases observed in Cory and P01. Overlapped rust inoculation with HL conditions did not further increase the effect induced by the HL treatment on stomatal conductance (Fig. 5, 6).

Photosynthetic parameters

HL treatment by itself slightly but significantly reduced the maximum quantum efficiency in all cvs except Cory, Selma and Alcudia (Fig 3, 7). However when HL treated plants were inoculated either with powdery mildew or rust a dramatic reduction of Fv/Fm ratios were observed in most genotypes. These reduction further decrease the already low Fv/Fm ratios due to powdery mildew alone in Charming, Orblanche and P01, and also the low Fv/Fm ratios induced by rust in Saia and Primula. There were not significant differences in the extent of the maximum quantum efficiency reduction of oats when superimposed powdery mildew or rust inoculation with HL ranging overall from 0.02 to 0.05 when overlapping HL and powdery mildew and from 0.02 to 0.06 when overlapping HL with rust. The barley P01 was by far the genotype most affected by the overlapping HL treatment with powdery mildew inoculation reducing its Fv/Fm ratio up to 0.40. In most cases, for both, powdery mildew and rust inoculated plants under HL, the reduction in Fv/Fm ratio were higher at longer times following inoculation, indicating a deterioration of the photosynthetic system with time.

The effect of HL treatment was not so obvious after inspecting the operating efficiency of PSII. Thus the oat cvs Araceli, Alcudia and Primula and the barley P01 plants grown under the moderately HL stress, significantly reduced their PSII operating efficiency whereas Charming, Cory, Orblanche, Kankan and Saia were not affected. Photoinhibition due to the HL conditions were only observed in the barley P01 genotype. However when overlapped HL with pathogen challenge different effects were observed. For instance Orblanche which show previously no effect following powdery mildew inoculation or HL treatment alone showed a significant reduction of the operating efficiency when challenge with the superimposed stresses and P01 showed a dramatic further decrease of the operating efficiency already reduced following *Bga* attack in parallel with a significant increase of photoinhibition. By contrary cvs Cory and Charming did not further decreased the already reduced operating efficiency following powdery mildew inoculation (Fig. 4). Interestingly, when overlapping rust and HL conditions all cvs significantly further reduced the already low operating efficiency induced by rust attack and increased the photoinhibition respect to the levels observed following inoculation alone.

DISCUSSION

Generality of effects and impact of physiological dysfunctions during plant resistance responses to pathogen attack

Our results show in oat important stomatal dysfunctions during the expression of resistance responses to powdery mildew and rust, confirming our previous work in barley (Prats *et al.*, 2006; Prats *et al.*, 2010; Prats *et al.*, 2007c) The pattern of the responses was also similar to that observed in barley. Thus, resistance responses to powdery mildew induced a reduction of diurnal stomatal conductance indicating a failure of stomata to open in light and a marked increase in nocturnal stomatal conductance. Resistance responses to rust induced a reduction in diurnal conductance and few changes in nocturnal stomatal conductance. Loss of stomatal ability to close in darkness have been also reported after the onset of HR in cucumber cotyledons infiltrated with avirulent bacteria (Popham *et al.*, 1993) and in a band of living epidermis surrounding necrotic lesions in potato leaf tissues killed by *Phytophthora infestans* infection (Farrell *et al.*, 1969) Thus, evidence from several different pathosystems indicates

that the death of cells induce stomatal dysfunctions. Opening and close of stomata provide the necessary balance to maintain the relative concentration of CO_2 for photosynthesis while preventing water losses (Roelfsema and Hedrich, 2005) Thus, one expected consequence of this altered stomatal behaviour would be a reduction in photosynthesis. In fact, we observed an extensive alteration on photosynthetic parameters leading to a reduced photosynthesis during execution of the resistance responses following *Bga* and *Pca* challenge. Interestingly, this reduction in photosynthesis would reduce the potential supply of assimilates available to pathogens, hence, limiting colony growth, sporulation and spreading of disease. Indeed, a recent study on *Xanthomonas axonopodis* pv*. citri,* shows that this pathogen encodes a plant-like natriuretic peptide (XacPNP) expressed specifically during the infection process to prevent reduction of photosynthesis. Challenge with the deletion mutant (ΔXacPNP) results in a mayor reduction of photosynthesis and a loss of infection success, suggesting that reducing photosynthesis is an effective plant defence mechanism against biotrophic pathogens (Garavaglia et al., 2010). These results also agree with recent studies on barley in which both HR (*Mla12*) and penetration resistance (*mlo*) based resistance responses severely inhibited photosynthesis (Swarbrick *et al.*, 2006) and highlight the importance of such phenomenon.

However our data do not only show a reduction on photosyntheis as a defence mechanism to counteract pathogen growth and preserve plant survival but plants highly compromised in its physiological functions showing even photoinhibition damages. Data suggest a disruption of stomatal behaviour consequence of execution of the resistance responses which could be directly linked to photosynthetic alterations, but also a direct impact on the photosintetic efficiency since genotypes only slightly affected on the stomatal behaviour such Saia or Alcudia shows important alterations in photosynthetic activity with implications for the whole leaf performance. This is supported by the high reduction on the Fv/Fm ratio. This ratio provides an estimate of the maximum quantum efficiency of PSII photochemistry (Butler, 1978) and has been widely use to detect stress-induced perturbations in the photosynthetic apparatus. Decreases in this ratio can be due to development of slowly relaxing quenching process and photodamage to PSII reaction centres, both of which reduce the maximum quantum efficiency of PSII photochemistry (Baker et al., 2004). In addition of the reduction of Fv/Fm ratio our data shows a general reduction in the F'q/F`m ratio or operating efficiency of PSII. Stomatal perturbations may alter the rate of ATP and NADPH consumption for $CO_2$ assimilation, which may result in decreases in the rate of linear electron flux and consequently in the F'q/F`m ratio. However operation of the water-water cycle and increase in photorespiration of C_3 plants such as oats may maintain rates of electron flux despite alterations in CO_2 therefore maintaining the F'q/F`m ratio (Baker et al., 2004). The high decreases in PSII operating efficiency observed in the resistant oat plants following pathogen challenge, both *Bga* and *Pca,* suggest either and excess of electron to cope with or a decrease in the buffering effect of electron sinks leading to the reduction in the F'q/F`m ratio. This could indicate further damage on the photosynthetic apparatus as discussed later. Indeed, increases in photoinhibition were observed in Cory and P01 and in Saia following *Bga* and *Pca* inoculation supporting this hypothesis.

Perturbation of stomatal behaviour also in the susceptible cvs could question the direct involvement of the resistance responses in the physiological dysfunctions observed. Obviously, disease development induces changes in plant metabolism. Earlier reports shows that powdery mildew (Majernik, 1971; Ayres and Zadoks, 1979) and rust (Scholes and Rolfe, 1996) disease induced stomatal and photosynthesis alterations. However, their measurements were done later during the infection period, several days after inoculation. In line with these results we also observed physiological alterations in the susceptible cvs. However the pattern of alterations was different to that observed in the resistant cvs. For instance increases in nocturnal stomatal conductance following powdery mildew inoculation was observed much

later than in the resistance cvs coinciding with the onset of sporulation and when senescence of the tissue was evident.

Are physiological dysfunctions determined by the extent of HR?

Our results suggest that there is not a direct link between the extent of the HR and the derived physiological, stomatal or photosynthetic, alterations. Thus, genotypes such as Cory, Orblanche and Charming with similar HR percentage showed different range of alterations in response to *Bga.* Charming showed a less intense lock-up, and lower reduction in maximum and operating PSII efficiency than Cory, and Orblanche showed intermediate levels. Similarly Alcudia and Kankan showed the same magnitude of HR in response to rust but Kankan showed further smaller physiological dysfunctions compared with Alcudia. This is of high practical importance since if for a similar resistance response there is a range of differently affected cvs at physiological level, this offer opportunity for breeding for this trait. Previous work on barley genotypes, P01, P02 and P23, differing in the timing or localisation of HR but with similar HR percentages, showed similar, in term of load, stomatal dysfunctions are following powdery mildew challenge (Prats *et al.*, 2010).These genotypes are isogenic lines suggesting that genetic background might in a certain manner influence the extent of the physiological dysfunctions. This suggestion is supported by previous studies in which stomatal dysfunction affected the barley genotype P22 but not Riso R, both *mlo5* but with different genetic background (Prats *et al.*, 2006). In addition, we also observed strong physiological alterations in the highly rust resistance cv Saia. in which resistance is based on resistance responses previous to mesophyll cell penetration and in which cell death was only very scarcely observed. This suggests that the physiological alterations relay not only in the death of the cells but also in a process triggered during both prepenetration and postpenetartion resistance. This is supported by our previous observations of stomatal dysfunction in the *mlo* barley P22 characterised by a high penetration resistance, albeit, they were associated with the moderate mesophyll cell death consequence of a second oxidative burst following papilla formation.

Influences of the pathogen life style on physiological dysfunctions

One of the major effects previously reported as consequence of the HR was the stomatal inability to close in the dark. As stomatal and subsidiary cells were turgid (Prats et al., 2010), this phenomenon cannot be explained as a loss of cell viability. A possible explanation is that epidermal cells have substantial influence on the turgor relations of the stomatal complexes (Roelfsema and Hedrich, 2005). When epidermal cells die as a result of HR, their collapse would reduce drastically their turgor relative to the stomatal complex and might reduce water flow to the subsidiary cells which would in turn lose turgor. If guard cell turgor is maintained by water flow from other routes (e.g. via living epidermal cells lying between complexes or via the mesophyll), alteration of turgor balance could cause the stomatal pore to open since opening depends on the balance between guard cell and subsidiary cell turgor (Roelfsema and Hedrich, 2005). If this would be true rust pathogen infecting mesophyll cells should provoke a differential effect on stomatal behaviour compared to that induced by powdery mildew attack on epidermal cells. In addition since epidermal cells usually lack photosynthetic activity, comparison on photosynthetic parameters following powdery mildew and rust attack might reveal further the importance of primary (physical) and secondary (physiological) effects.

Based on the response observed after epidermal and mesophyll cell death following powdery mildew and rust attack respectively we suggest a complex interplay between primary and secondary effects reflecting the epiphytic style of mildew versus the initial

epiphytic location of rust, which is followed by endophytic attack in mesophyll cells. The marked stomatal dysfunctions observed during oat-powdery mildew interaction suggest an important role for the proximity of dead epidermal cells in stomatal behaviour, this effect was not so accentuated following mesophyll cell death although rust appresoria formation over stomata and appressorial hyphae growing through stomatal pore into substomatal vesicle could influence the response observed following rust attack. This physical/physiological stomatal disruption would be an important component of the altered photosynthetic parameters that could contribute to the higher impact observed after powdery mildew challenge compared with rust. In addition powdery mildew attack on epidermal cells induced alterations on mesophyll cell photosynthetic activity far beyond those expected according to the stomatal dysfunction indicating a possible damage of the photosynthetic machinery on these cells.

Are physiological dysfunctions related to the ability to cope with increasing oxidative stress induced by resistance responses?

The lack of correlation between the extent of physiological dysfunctions and percentage of dead cells and between altered photosynthethic activity and stomatal behaviour suggest an involvement of other components contributing the observed perturbations. It is known than an oxidative burst is one of the components of the cell death based resistance mechanisms (Lamb and Dixon, 1997). The reactive oxygen species generated are not only direct protective agents, but also functions as a substrate for oxidative cross-linking in the cell wall, as a threshold trigger for hypersensitive cell death, and as a diffusible signal for induction of cellular protectant genes in surrounding cells. Thus ROS generation is not only involved in HR development but in an overall resistance response. Indeed, H_2O_2 generation has been reported in barley at *Bgh* attack sites related with efficient papilla deposition (Piffanelli *et al.*, 2004). Thus, an excess of reactive oxygen species generation and/or the inability to detoxify the ROS produced during the resistance response to powdery mildew might be involved in the physiological alterations observed. To test this hypothesis we superimposed pathogen challenge with a moderate light stress since excess light also leads to ROS production. Our data show moderate stomatal and photosynthetic alterations in most cvs. following the light stress but more important, overlapping pathogen attack, either powdery mildew or rust, boots the impact on the physiological functions by far compared with effect of inoculation or light stress alone. This suggests that excess of ROS produced during the development of the resistance responses to pathogen or the inability to cope with it might be responsible of the physiological dysfunctions and hence might be a component of the resistance cost. ROS quantification and the antioxidant capacity of the different oat cvs, are currently being investigated to further confirm this hypothesis.

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Tables

Table 1: Microscopic assessment of fungal development and leaf epidermal cell responses following inoculation with *B. graminis* f. sp. *avenae* and *P. coronata* f.sp. *avenae*.

Genotype	**Penetration resistance**	**Early HR**	**Late HR**	**Established colonies**
Blumeria gramisnis f.sp. *avenae* inoculation				
Selma	21.5+3.0	0.0+0.0	0.0+0.0	78.5+3.0
Cory	44.8+2.5	37.5+3.5	10.5+0.5	7.3+1.4
Charming	46.0+6.7	22.0+5.1	28.2+5.4	3.8+0.9
Orblanche	37.3+4.8	16.8+2.7	25.0+2.9	21.0+4.3
P01 (barley)	47.3+0.5	47.0+3.1	0.0+0.0	5.75+2.9
l.s.d.*	6.71	6.09	4.91	7.87
Puccinia coronata f.sp. *avenae* inoculation				
Araceli	42.1+4.0	0.0+0.0	0.0+0.0	57.8+4.0
Alcudia	64.8+3.8	15.8+2.3	15.9+1.5	3.5+0.8
Kankan	55.4+1.7	5.7+2.4	23.0+2.1	15.8+0.8
Primula	46.7+3.1	0.0+0.0	17.0+2.4	36.3+0.8
Saia	85.0+0.4	7.7+1.4	0.0+0.0	7.3+1.3
l.s.d.*	5.24	5.76	3.35	4.72

*Least significance differences ($p<0.05$) for statistical comparisons

FIGURE CAPTION

Figure 1. A) Leaf water conductance (g_1) of healthy (open) and inoculated (solid) Cory, Charming, Orblanche, Selma and P01 leaves with *Blumeria graminis* f. sp. *avenae* assessed at mid-light period during a 8 days' time course, when growing under two different light regimes, low light intensity (circles) and high light intensity (triangles). B) Area Under the Conductance Progress Curve (AUCPC) of healthy (Cont) and inoculated (Inoc) Cory, Charming, Orblanche, Selma and P01 leaves with *Blumeria graminis* f. sp. *avenae* for the 8 days' time course, when growing under two different light regimes, low light intensity (LL) and high light intensity (HL). Errors Bars indicate standar error.

Figure 2. A) Leaf water conductance (g_1) of healthy (open) and inoculated (solid) Cory, Charming, Orblanche, Selma and P01 leaves with *Blumeria graminis* f. sp. *avenae* assessed 1h before the end of each dark period, during a 8 days' time course, when growing under two different light regimes, low light intensity (circles) and high light intensity (triangles). B) Area Under the Conductance Progress Curve (AUCPC) of healthy (Cont) and inoculated (Inoc) Cory, Charming, Orblanche, Selma and P01 leaves with *Blumeria graminis* f. sp. *avenae* for the 8 days' time course, when growing under two different light regimes, low light intensity (LL) and high light intensity (HL). Errors Bars indicate standar error.

Figure 3. A) Time course of Maximum Quantum Yield (Fv/Fm) of healthy (open) and inoculated (solid) Cory, Charming, Orblanche, Selma and P01 leaves with *Blumeria graminis* f. sp. *avenae* assessed at mid-light period during a 8 days' time course, when growing under two different light regimes, low light intensity (circles) and high light intensity (triangles).

Figure 4. PSII operating efficiency, F_q'/F_m (left) and photoinhibitory quench, q_I (right) of Cory, Charming, Orblanche, Selma and P01 of healthy (Cont) and inoculated (Inoc) Cory, Charming, Orblanche, Selma and P01 leaves with *Blumeria graminis* f. sp. *avenae* when growing under two different light regimes, low light intensity (LL) and high light intensity (HL). Errors Bars indicate standar error.

Figure 5. A) Leaf water conductance (g_1) of healthy (open) and inoculated (solid) Cory, Charming, Orblanche, Selma and P01 leaves with *Puccinia coronata* f. sp. *avenae* assessed at mid-light period during a 8 days' time course, when growing under two different light regimes, low light intensity (circles) and high light intensity (triangles). B) Area Under the Conductance Progress Curve (AUCPC) of healthy (Cont) and inoculated (Inoc) Cory, Charming, Orblanche, Selma and P01 leaves with *Blumeria graminis* f. sp. *avenae* for the 8 days' time course, when growing under two different light regimes, low light intensity (LL) and high light intensity (HL). Errors Bars indicate standar error.

Figure 6. A) Leaf water conductance (g_1) of healthy (open) and inoculated (solid) Cory, Charming, Orblanche, Selma and P01 leaves with *Puccinia coronata* f. sp. *avenae* assessed 1h before the end of each dark period, during a 8 days' time course, when growing under two different light regimes, low light intensity (circles) and high light intensity (triangles). B) Area Under the Conductance Progress Curve (AUCPC) of healthy (Cont) and inoculated (Inoc) Cory, Charming, Orblanche, Selma and P01 leaves with *Puccinia coronata* f. sp. *avenae* for the 8 days' time course, when growing under two different light regimes, low light intensity (LL) and high light intensity (HL). Errors Bars indicate standar error.

Figure 7. A) Time course of Maximum Quantum Yield (Fv/Fm) of healthy (open) and inoculated (solid) Cory, Charming, Orblanche, Selma and P01 leaves with *Puccinia coronata* f. sp. *avenae* assessed at mid-light period during a 8 days' time course, when growing under two different light regimes, low light intensity (circles) and high light intensity (triangles).

Figure 8. PSII operating efficiency, F_q'/F_m (left) and photoinhibitory quench, q_I (right) of Cory, Charming, Orblanche, Selma and P01 of healthy (Cont) and inoculated (Inoc) Cory, Charming, Orblanche, Selma and P01 leaves with *Puccinia coronata* f. sp. *avenae* when growing under two different light regimes, low light intensity (LL) and high light intensity (HL). Errors Bars indicate standar error.

FIGURES

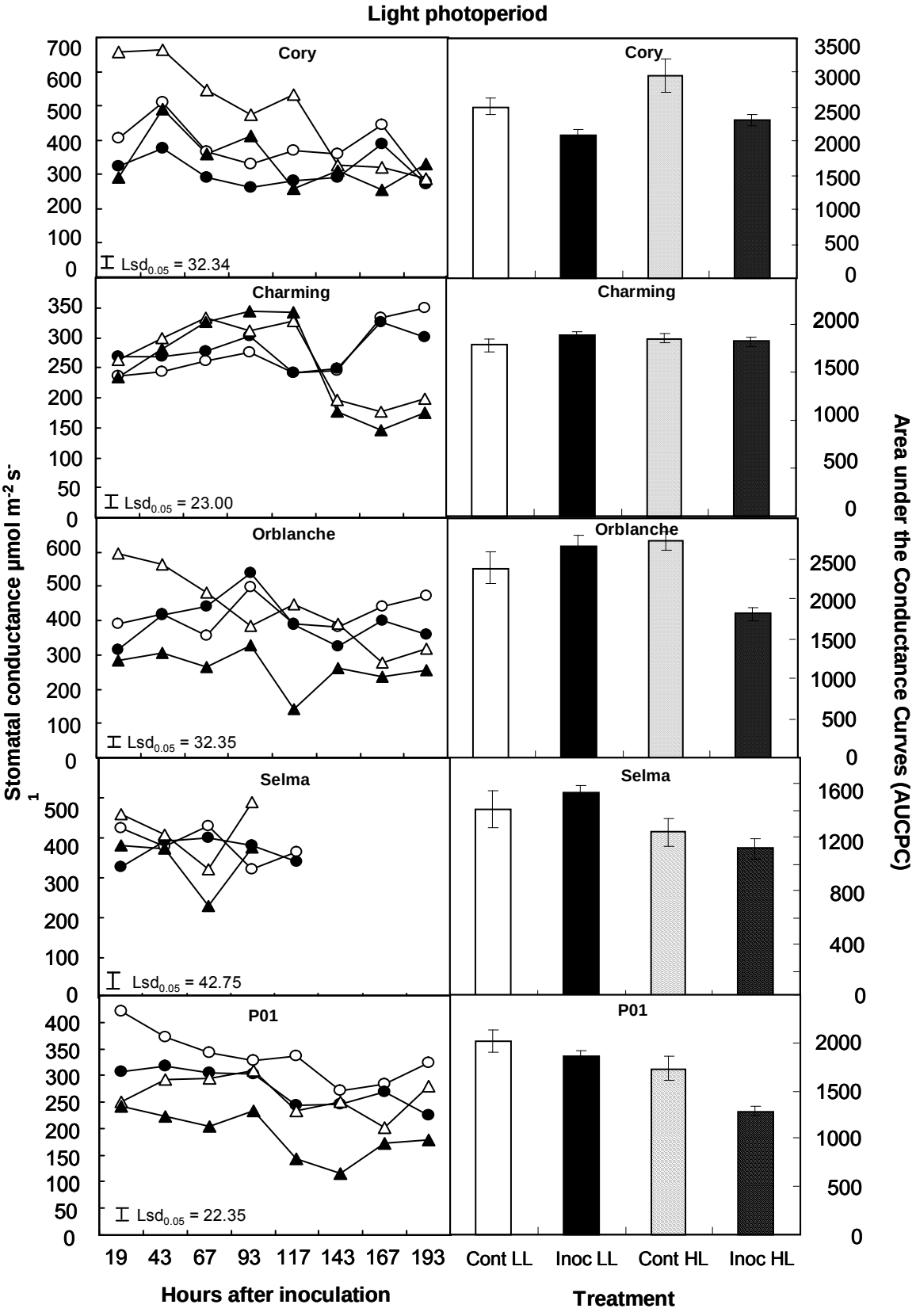

Figure 1.

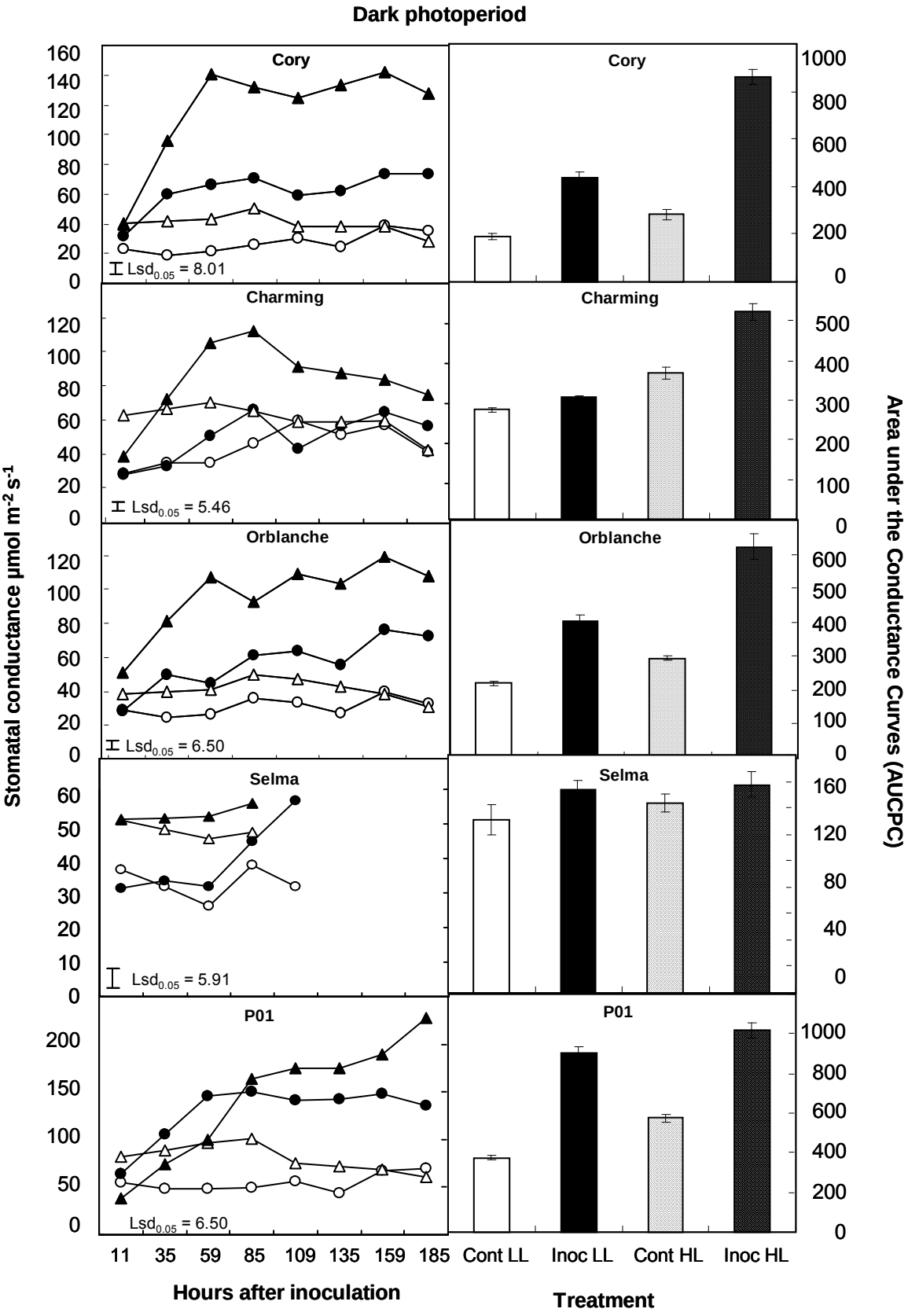
Dark photoperiod
Cory
Charming
Orblanche
Selma
P01
Lsd0.05 = 8.01
Lsd0.05 = 5.46
Lsd0.05 = 6.50
Lsd0.05 = 5.91
Lsd0.05 = 6.50
Stomatal conductance µmol m-2 s-1
Area under the Conductance Curves (AUCPC)
11 35 59 85 109 135 159 185
Hours after inoculation
Cont LL
Inoc LL
Cont HL
Inoc HL
Treatment

Figure 2.

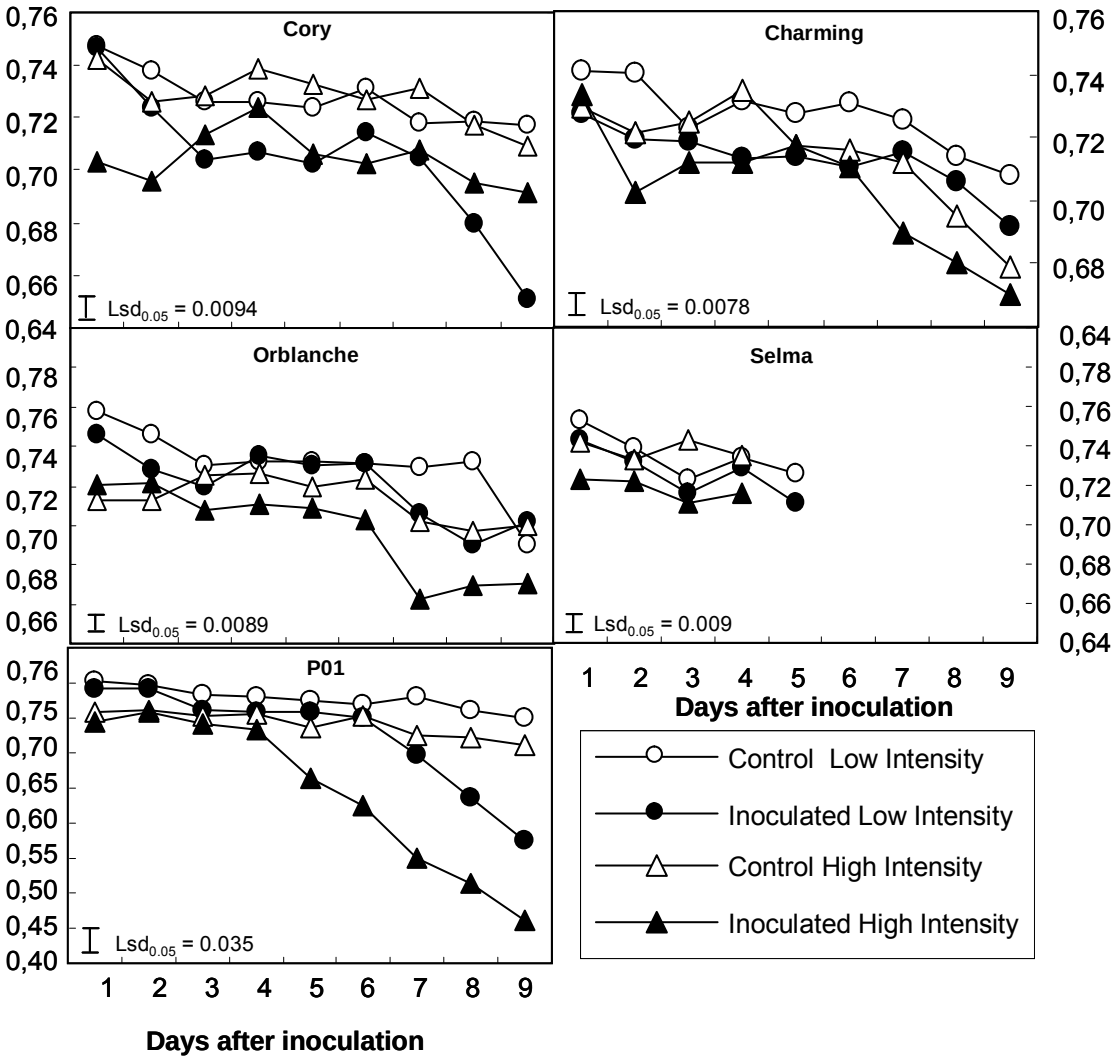
Cory
Charming
Orblanche
Selma
P01
Lsd0.05 = 0.0094
Lsd0.05 = 0.0078
Lsd0.05 = 0.0089
Lsd0.05 = 0.009
Lsd0.05 = 0.035
Days after inoculation
Control Low Intensity
Inoculated Low Intensity
Control High Intensity
Inoculated High Intensity

Figure 3.

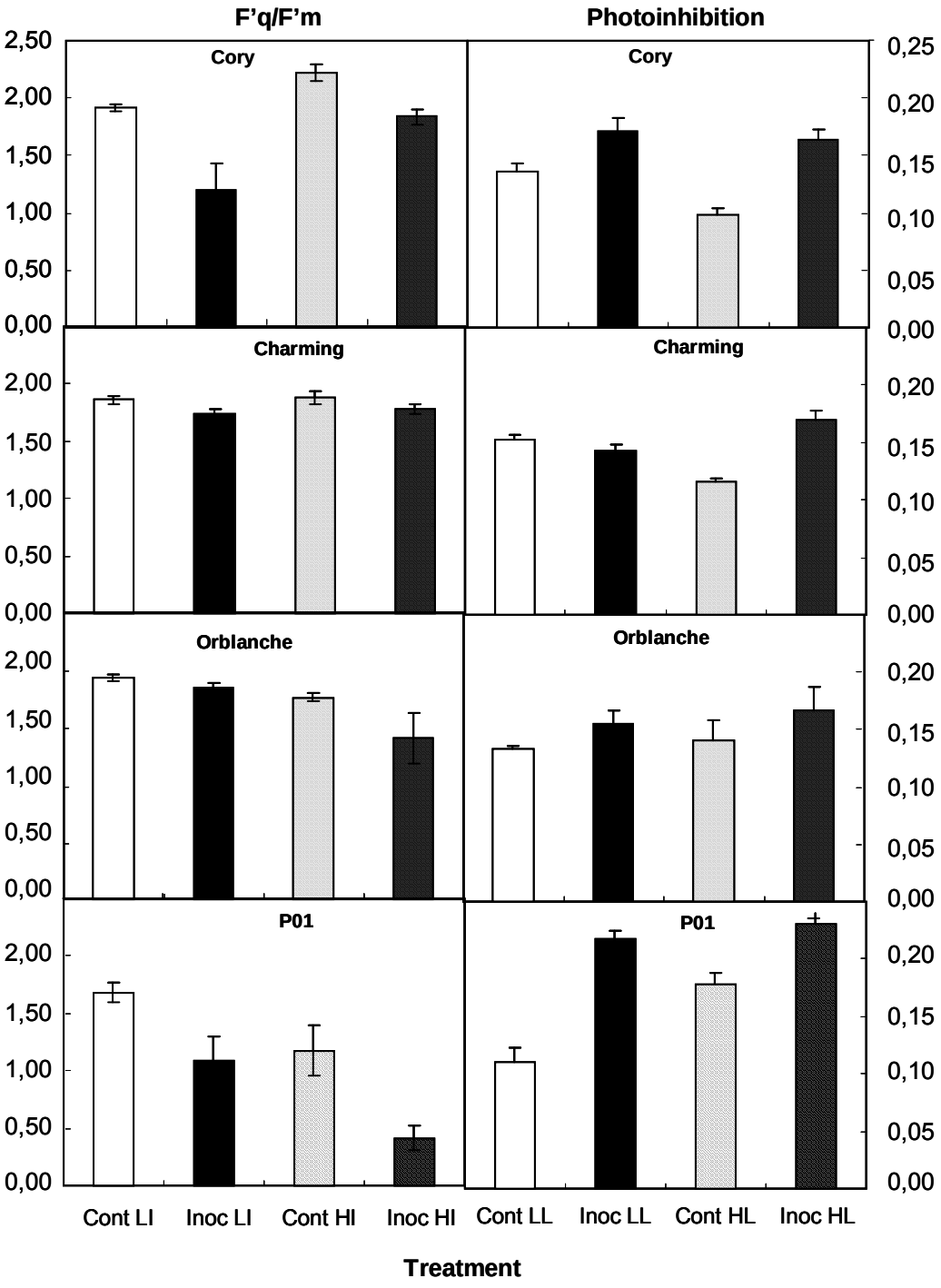
F'q/F'm
Photoinhibition
Cory
Charming
Orblanche
P01
2,50
2,00
1,50
1,00
0,50
0,00
0,25
0,20
0,15
0,10
0,05
Cont LI
Inoc LI
Cont HI
Inoc HI
Cont LL
Inoc LL
Cont HL
Inoc HL
Treatment

Figure 4.

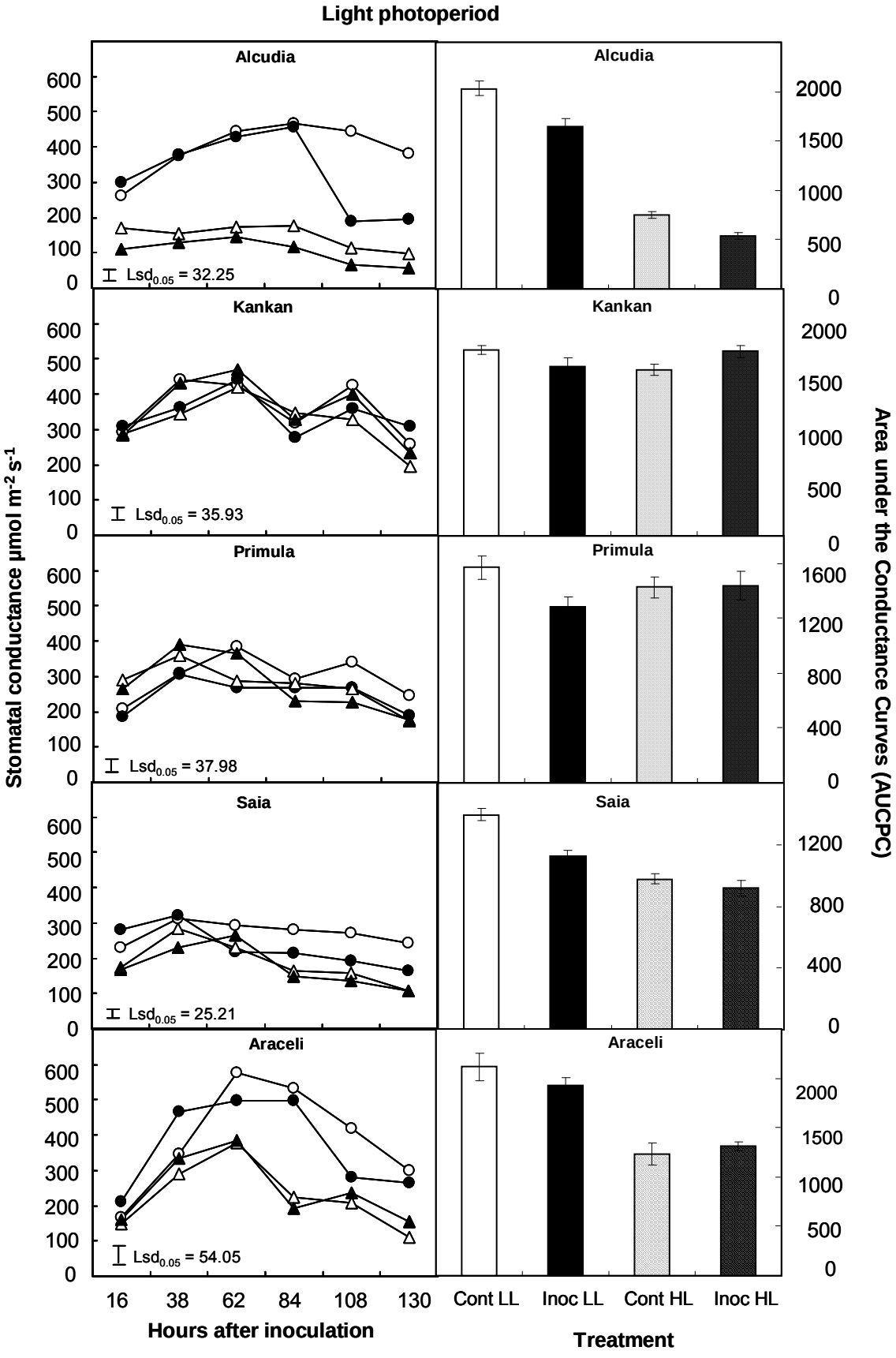
Light photoperiod
Alcudia
Kankan
Primula
Saia
Araceli
Lsd0.05 = 32.25
Lsd0.05 = 35.93
Lsd0.05 = 37.98
Lsd0.05 = 25.21
Lsd0.05 = 54.05
Stomatal conductance µmol m-2 s-1
Area under the Conductance Curves (AUCPC)
16
38
62
84
108
130
Hours after inoculation
Cont LL
Inoc LL
Cont HL
Inoc HL
Treatment

Figure 5.

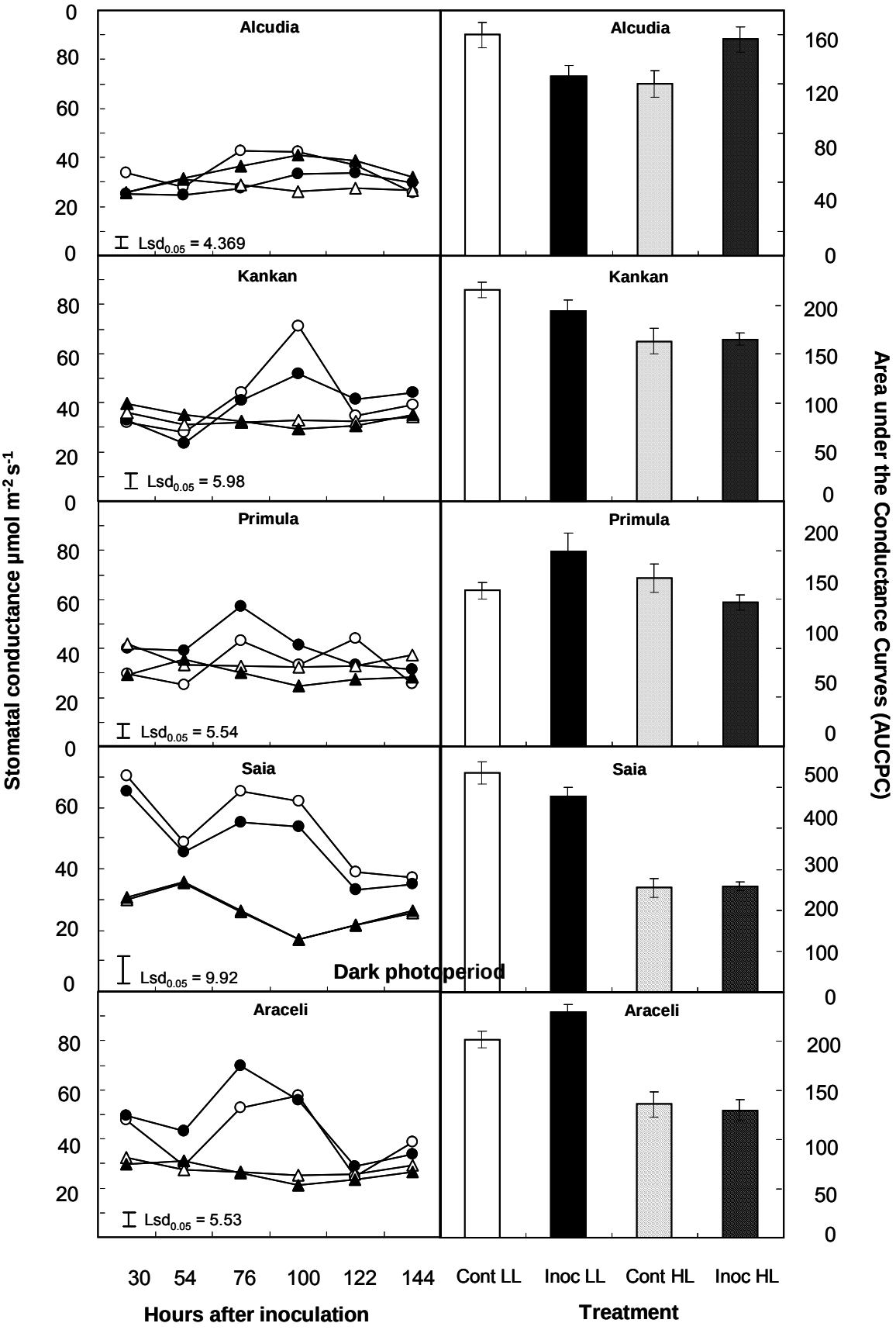
Alcudia
Kankan
Primula
Saia
Araceli
Lsd0.05 = 4.369
Lsd0.05 = 5.98
Lsd0.05 = 5.54
Lsd0.05 = 9.92
Lsd0.05 = 5.53
Dark photoperiod
Stomatal conductance µmol m-2 s-1
Area under the Conductance Curves (AUCPC)
30 54 76 100 122 144
Hours after inoculation
Cont LL Inoc LL Cont HL Inoc HL
Treatment

Figure 6.

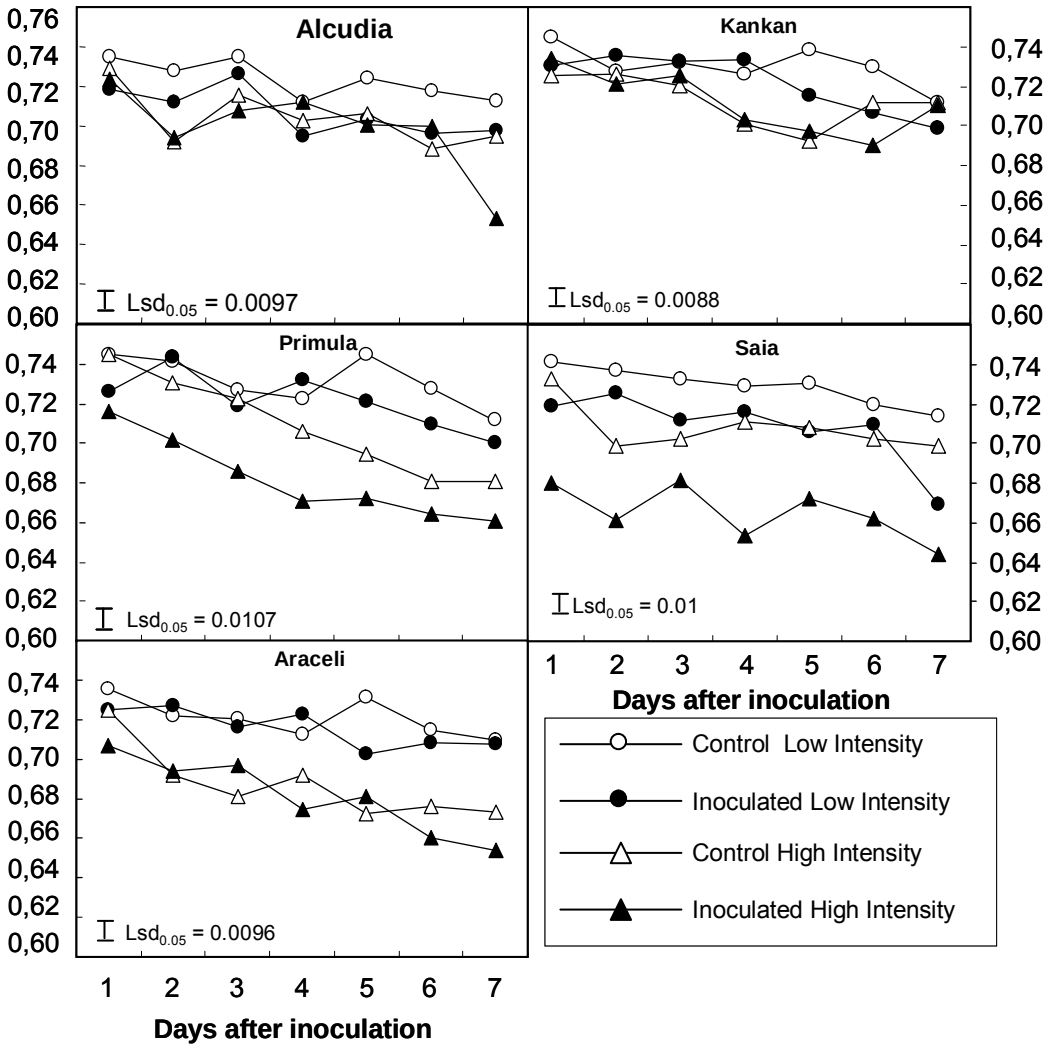
Alcudia
Kankan
Primula
Saia
Araceli
Lsd0.05 = 0.0097
Lsd0.05 = 0.0088
Lsd0.05 = 0.0107
Lsd0.05 = 0.01
Lsd0.05 = 0.0096
0,76
0,74
0,72
0,70
0,68
0,66
0,64
0,62
0,60
1 2 3 4 5 6 7
Days after inoculation
Control Low Intensity
Inoculated Low Intensity
Control High Intensity
Inoculated High Intensity

Figure 7.

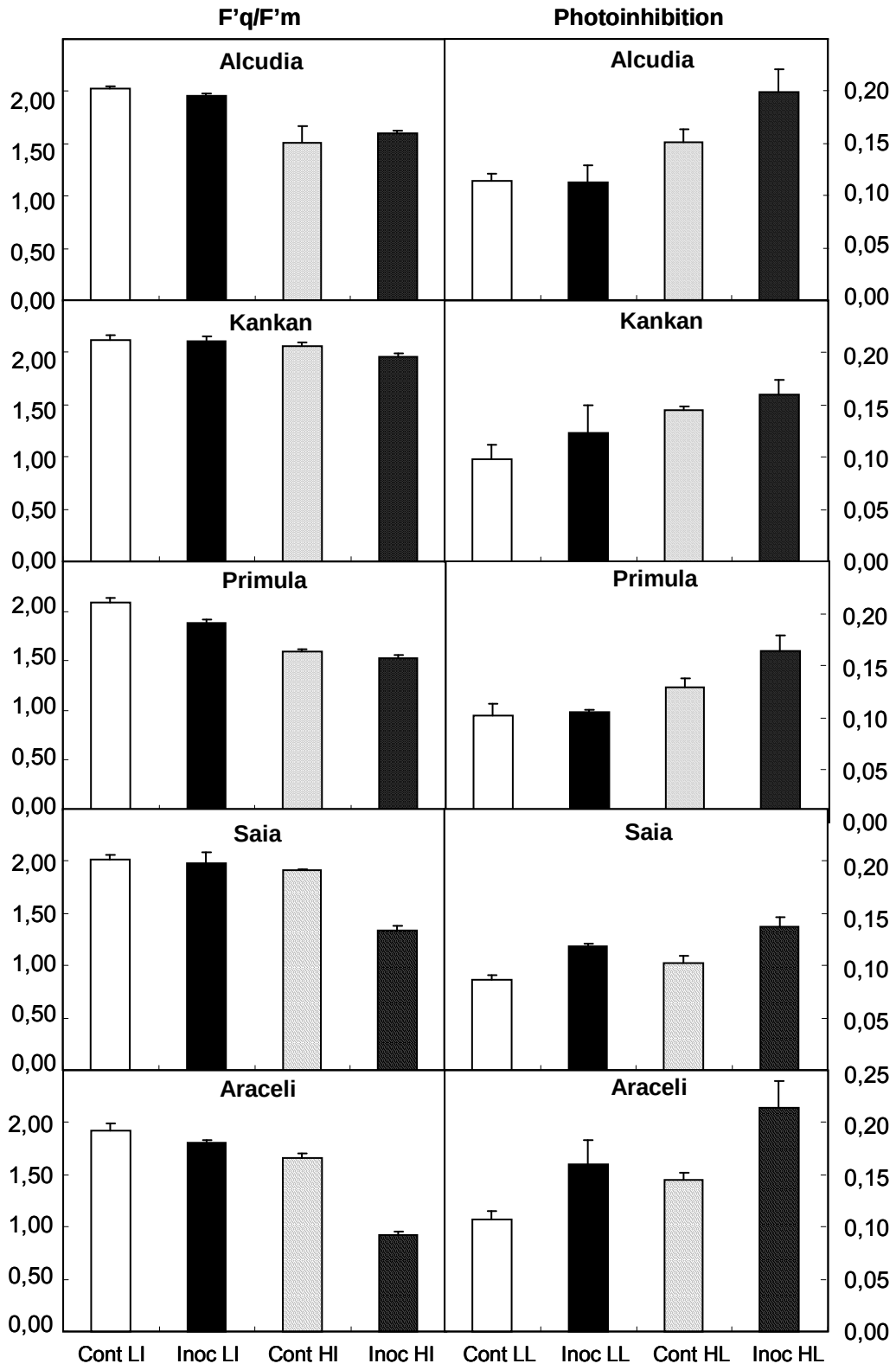
F'q/F'm
Photoinhibition
Alcudia
Kankan
Primula
Saia
Araceli
Cont LI
Inoc LI
Cont HI
Inoc HI
Cont LL
Inoc LL
Cont HL
Inoc HL

Figure 8.

Targeting sources of drought tolerance within an *Avena* spp collection through multivariate approaches.

Javier Sánchez-Martín[1], Luis AJ Mur[2], Diego Rubiales[1], Elena Prats[1].*

[1]CSIC, Institute for Sustainable Agriculture, Apdo. 4084, E-14080 Córdoba, Spain. JSM:
[2]Institute of Biological, Environmental and Rural Sciences, University of Aberystwyth, UK;

*For correspondence E-mail: elena.prats@ias.csic.es
Tel: +34 957499291

Fax: +34 957 499252

ABSTRACT

In this study we found and characterised sources of tolerance to drought amongst an oat (*Avena sativa* L.) germplasm collection of 174 landraces and cultivars. Further we used multivariate analysis to suggest the key mechanism/s responsible for coping with drought stress. Following initial assessment of drought symptoms and area under the drought progress curve a subset of 14 accessions were selected for further analysis. The collection was assessed for relative water content, cell membrane stability, stomatal conductance, leaf temperature, water use efficiency, lipid peroxidation, lipoxygenase activity, chlorophyll levels and antioxidant capacity during a drought time-course experiment. It proved difficult to unequivocally link drought tolerance to specific physiological processes in the different resistant oat accessions. However, multivariate statistical approaches indicated that two traits involved in water relations such as leaf temperature and relative water content, together with the area of drought curves were the major source of drought tolerance being the most important discriminatory parameters. Other parameters involved in water use such as stomatal conductance and water use efficiency were not so useful to discriminate among accessions. More importantly, these analyses allowed for the first time the ranking of many supposed drought tolerance traits in order of degree of importance within this crop thereby highlighting those with a causal relationship to drought stress tolerance. Further, this work represented a cost-effective initial screen which could be subsequently employed to target drought tolerance amongst lines in segregating populations.

Keywords: *Avena sativa* oat, drought, tolerance mechanisms, multivariate approaches.

Abbreviations.

AUCPC	Area under the conductance progress curve
AUDPC	Area under the drought progress curve
CMS	Cell membrane stability
daww	Days after withholding water
DFA	Discriminant function analysis
g_1	Stomatal conductance
IR	Infrared
LOX	Lipoxigenase activity
LP	Lipid peroxidation
PCA	Principal components analysis
RWC	Relative water content
SCMR	SPAD chlorophyll meter reading
WUE	Water use efficiency

INTRODUCTION

Oat (*Avena sativa* L.) rank at around sixth in world cereal production statistics, following wheat, maize, rice, barley and sorghum (FAO, 2011). It is widely grown in temperate areas, with an increasing interest to expand the crop to subtropical areas, Mediterranean countries (Stevens *et al.*, 2004) and northeast China (Islam *et al.*, 2011). This is mainly due to its good adaptation to a wide range of soil types and because on marginal soils oats can perform better than other small-grain cereals (Stevens *et al.*, 2004). However, oats can be sensitive to hot, dry weather and hence, in most Mediterranean and dry regions drought is the main limiting factor for oat yield probably exceeding losses from all other causes (Stevens *et al.*, 2004). Thus, new sources of oat tolerance must be exploited which may be introgressed into elite cultivars.

Plant breeding has an important role in improving germplasm to fit the agroclimatic conditions of drought-prone areas (Chaouki *et al.*, 2004). However, cereal breeding in general, and oat breeding in particular, has been mainly based on empirical selection for yield but this is characterized by a low heritability and a high genotype x environment interaction making it a poor assessment criterion (Araus *et al.*, 2002). As a result, modern breeding strategies attempt to include assessments of physiological, biochemical and molecular characteristics which may better reflect lineage productivity and responses to environmental stress (Araus, 1996; Richards, 1996; Slafer and Araus, 1998). A corollary of this approach is a better understanding of drought tolerance mechanisms which in turn will further define targets in germplasm screens.

Several morphological, physiological and molecular plant responses can contribute for coping with drought stress either increasing its ability to avoid damage (avoidance mechanisms) and/or to maintain its metabolic functions under water limiting conditions (tolerance mechanisms). In this work we focussed in the tolerance mechanisms, where the physiological bases of genetic variation are far from being clear. Key features may be the capacity to maintain cell/tissue water, cell membrane stability, and to avoid oxidative damage through antioxidant machinery (Farooq *et al.*, 2009). Thus, water related features such as relative water content (RWC), leaf water potential (LWP), stomatal conductance (g_1), transpiration rate and leaf/canopy temperature have been studied in different species under drought stress. In general water stressed plants have lower relative water content, leaf water potential and transpiration rate with a concomitant increase in leaf temperature. Furthermore, a positive correlation between grain yield and RWC have been observed in durum and bread wheat (Merah, 2001, Singh and Patel, 1996), Particularly in oat, decreases in g_1, in the difference between air and leaf temperatures and in RWC were associated to water deficit. However none of these indices by itself was associated with degree of yield losses (Peltonen-Sainio and Makela, 1995).

Improved tissue water status may be also achieved through osmotic adjustment. This involve 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) which allows the cell to decrease osmotic potential and hence increase the gradient for water influx and turgor. Thus, osmotic adjustment has been related to grain yield under water deficit environments (Moinuddin et al., 2005) and considered as a selection criterion for drought tolerance in wheat (Morgan, 1983).

In addition to water related features, physiological traits indicative of oxidative damage and antioxidant defence have been well documented under drought stress conditions (Farooq *et al.*, 2009) and references therein). Altogether these changes are thought to be associated with protecting cellular functions or with maintaining the structure of cellular components (Seki *et al.*, 2007). Particularly in oat it has been shown that as part of acclimation to drought stress, the lipid

composition of root plasma membranes is selectively modified, possibly to increase their flexibility (Larsson et al., 2006). Molecular targets contributing drought tolerance, including changes in gene expression, synthesis of stress proteins and activation of molecular signalling have been only recently dissected disclosing the intricate complexity of the resistance responses to this stress (Farooq *et al.*, 2009; Seki *et al.*, 2007; Kavar *et al.*, 2008).

This complexity is an important handicap for breeding. Efforts have been made to produce drought tolerant genotypes based on the knowledge of plant responses to drought and the mechanisms involved described above. However these were not always successful since although changes in water related features have been described during drought stress, not all of these features are suitable for discriminating tolerant from sensitive genotypes and not all plant species respond in a similar manner. For instance, leaf water potential was able to discriminate drought resistant and susceptible barley cultivars (Matin et al., 1989) and it have been reported to be the main trait responsible of the drought tolerance phenotype in chickpea which allowed its use as phenotypic marker in breeding programmes (Pannu *et al.*, 1993); however, it is not a defining feature of tolerance in bread wheat (Schonfeld et al., 1988) or faba bean (Ricciardi *et al.*, 2001). On the other hand, in bread wheat, RWC differed significantly among susceptible and resistant populations under increasing drought (Schonfeld et al., 1988). Thus, it remains uncertain in a given species which is the best feature(s) indicative of drought tolerance and/or when these should be assessed. In the present study in addition to seeking to characterise new sources of drought tolerance, we use multivariate analysis in a range of drought linked features and genotypes to reveal the key physiological mechanism/s in the oats for coping with drought stress.

MATERIALS AND METHODS

Plant material and treatments

For the resistance screening we used a germplasm collection of landraces consisting in 107 Spanish accessions of *A. sativa* L. and 31 of *A. byzantina* K. Koch kindly provided by the "Centro de Recursos Fitogenéticos", INIA, Madrid, Spain, and 36 commercial cultivars supplied by the Andalusian Network of Agriculture Experimentation (RAEA). For easier comparison among landraces and manuscript reading, germplasm bank codes were substituted for others codes easier to read (Sánchez-Martín *et al.*, 2011). Oat cultivars studied were: Ac1, Acebeda, Adamo, Aintree, Alcudia, Anchuela, Araceli, Brawi, Caleche, Cannele, Chambord, Chappline, Charming, Cobeña, Condor, Cory, Edelprinz, Flega, Fringante, Fuwi, Hammel, Kankan, Kantora, Karmela, Kassandra, Kazmina, Mirabel, Mojacar, Norly, Orblanche, Pallini, Patones, Prevision, Primula, Rappidena and Saia. Seedlings were grown in 0.5 L pots filled with peat:sand (3:1) in a growth chamber with 20 ºC, 65 % relative humidity and under 12 h dark/12 h light with 150 µmol m^{-2} sec^{-1} photon flux density supplied by high-output white fluorescent tubes (OSRAM, Spain).

Plants were grown for 21 days until second leaf was fully expanded (their third-formed leaf was unrolled). During this time trays carrying the pots were freely watered with a thin layer of water of approximately 1 cm continuously present in the tray. Then in those plants subjected to drought, water was withheld for 19 days at which experiment finished. Control plants were watered as above mentioned during the whole experiment.

Additionally selected accessions were growth during the 2010-2011 crop season in a field plot in Salamanca, Spain (40º 55' 28.2'' N, 5º 21' 45.54''W) in water prone conditions (although not severe stress) i.e. 242.36 mm water in the growth season compared with the 600 mm by mean of north European oat growing areas. The soil of the experimental field, around 0.8 m deep, is a sandy loam or sandy-clay-loam Vertic Luvisol (FAO2011). Plants were sown on 01/11/2010. Each accession/replication was represented by 3 rows of 1 m long, with each row consisting of 30 plant.

The distance between rows was 0.5 m. Three replicates were grown in a randomized complete block design.

Visual assessment of drought symptoms

From the time at which water was withheld for drought treatment (from now on T_0) all plants were visually evaluated daily according to the following scale: 0 = vigorous plant, no leaves shows drought symptoms; 1 = one or two leaves show slight drought symptoms (less turgor) but most leaves remain erect; 2 = most leaves show slight levels of drought stress, however one or two leaves still show no drought symptoms; 3 = all leaves show drought symptoms but these are no severe; 4 = all leaves show severe drought symptoms including incipient wilting; 5 = the whole plant is wilted with all leaves starting to dry, rolled and or shrunken (Online Resource 1). Five plants per accession were assessed. Drought severity values daily assessed according to this scale were used to calculate the area under the drought progress curve (AUDPC) for each oat accession similarly to the area under the disease progress curve widely used to disease screenings (Jeger and Viljanen-Rollinson, 2001) using the formula:

$$AUDPC= \sum_k^{i=1} ½ [(S_i+S_{i+1})(t_{i+1}-t_i)]$$

where S_i is the drought severity at assessment date i, t_i is the number of days after the first observation on assessment date i and k is the number of successive observations.

Relative water content

RWC was measured in ten plants per accession according to (Barrs and Weatherly, 1962). Measurements were carried out in the second leaves at time 0, 6, 9, 12, 15 and 18 days after withholding water (daww). Six hours after the onset of the light period, leaf blade segments were weighed (fresh weight; FW), floated on distilled water at 4 ºC overnight and weighed again (turgid weight; TW). They were then dried at 80 ºC for 48 h. After this, the dry weight (DW) was determined. RWC was then calculated as $RWC = (FW - DW) (TW - DW)^{-1} \times 100$.

Cell membrane stability

CMS was measured in ten plants per accession according to (Tripathy *et al.*, 2000). Measurements were carried out in the second leaves at 0, 6, 9, 12, 15 and 18 daww Samples collected were washed three times in deionized water to remove electrolytes adhered on the surface. The samples were then kept in a capped vial (20 ml) containing 10 ml of deionized water and incubated in the dark for 24 h at room temperature. The conductance was measured with a conductivity meter (CMD 510, WPA, UK). After the first measurement the vials were autoclaved for 15 min to kill the leaf tissue and release the electrolytes. After cooling, the second conductivity reading was taken. These two measurements were carried out individually for all the samples from both the control and stress treatments. The control gave a measure of leakage solely due to the cutting and incubation of leaf discs. The conductance of the stress sample was a measure of electrolyte leakage due to water stress and was assumed to be proportional to the degree of injury to the membranes. CMS was calculated as the reciprocal of cell-membrane injury after Blum & Ebercon, (1981): CMS% = [(1-(T1/T2)) / (1-(C1/C2))] x 100, where T and C refer to the treated and control samples, respectively; the subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

Stomatal conductance

g_l was measured in ten plants per accession with an AP4 cycling porometer (Delta-T Devices Ltd, Cambridge, UK). g_l is the sum of epidermal and stomatal conductance, but as epidermal conductance of oat is low, changes in g_l largely reflect changes in stomatal aperture. The porometer allows rapid measurement that is non-destructive and samples a relatively large area (17.5 x 2.5 mm) of leaf. It was used on the centre of the adaxial surface of leaf laminae. Measurements were carried out in the second leaves during the first 10 daww After this period stomata were strongly closed at all hours of the day with similar readings of that at dark period. Measurements were taken three times per day, two hours after onset of the light period, at the middle of the light period and two hours before the onset of the dark period. The area of the conductance progress curve (AUCPC) of drought treated plants with respect to the control curve of non-stressed plants was calculated using the formula above mentioned for AUDPC.

Infrared temperature

Leaf temperature was estimated on the second leaves of five plants per accession, using an infrared camera (FLIR i50, FLIR Systems Inc,). The final measurement of each plant was the mean of four measurements per leaf. Measurements were taken 6 hours after the onset of the light period at 6, 12 and 18 daww in control and stressed plants.

Water use efficiency

Water use efficiency (WUE) expressed in terms of plant production per water consumed was measured gravimetrically in 5 plants of each of the 14 selected accessions according to (Xin *et al.*, 2008). Briefly, pots were filled with the above mentioned substrate and watered until water dripped from the bottom. Three seeds were planted per pot and thinned to one plant at 7 days after emergence. The pots were then covered from both ends with 2 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 five weeks) pot weight was 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 80ºC when the samples reached a constant weight. WUE was calculated by dividing the total dry biomass by the amount of water transpired.

Lipid peroxidation and lipoxygenase activity

Lipid peroxidation and lipoxygenase activity was measured in five plants per accession in the second leaves of control and stressed plants at time 9 and 12 daww

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content following the method of (Rosales *et al.*, 2006) with slight modification. For the assay of MDA, second leaves were ground with a mortar and pestle with liquid nitrogen, homogenized in 50 mmolL^{-1} potassium phosphate buffer (pH 6.0) (1:5 (p/v)) and centrifuged at 20 000 × *g* for 25 min at 4ºC. For measurement of MDA content, 200 µl of 200 g L^{-1} trichloroacetic acid containing 5 g L^{-1} thiobarbituric acid was added to 50 µl aliquots of the supernatant. The mixture was heated at 95 ºC for 30 min and then quickly cooled in an ice-bath. Subsequently, the samples were centrifuged at 10 000 × *g* for 10 min at 4 ºC and the absorbance of the supernatant was read at 532 nm. The value for the non-specific absorption at 600 nm was subtracted from the A_{532} reading. The concentration of MDA was calculated using a calibration curve (0.1-1.2 µg μL^{-1}) of MDA.

Lipoxygenase enzyme (LOX, EC 1.13.11.12) activity was measured according to (Minguez-Mosquera *et al.*, 1993) using 50 mmol L^{-1} potassium phosphate buffer (pH 6.0) for extraction. The reaction mixture carried out in microwells consisted of 285 µl of 50 mmol L^{-1} potassium phosphate buffer (pH 6.0), 10µL of crude extract and 5 µl of 0.5 mmol L^{-1} linoleic acid in 50 mmolL^{-1} potassium phosphate buffer (pH 6.0). The LOX activity was calculated following the increase in the extinction at 234 nm using an extinction coefficient of 25 000 Lmol^{-1}. For preparation of substrate, linoleic acid (0.5 g) of higher than 99 % purity (Sigma) and 0.5 g Tween 20 were dissolved in deionized and deoxygenated H_2O. In the case of turbidity, a few drops of 2 M NaOH were added until complete transparency. The final volume of the mixture was taken to 25 mL. Aliquots of 2 ml were put into flasks which were closed under N_2.

Leaf chlorophyll content

Leaf chlorophyll was indirectly estimated on the second leaves of five plants per accession, using a SPAD-502 chlorophyll meter (Minolta Co., LTD., Japan) (Zhao et al., 2010). The final measurement of each plant was the mean of three measurements per leaf, the adaxial side of the leaves was always placed toward the emitting window of the instrument. Measurements were taken 6 hours after the onset of the light period at 6, 9, 12, 15 and 18 daww in control and stressed plants.

Antioxidant activity

Antioxidant activity was measured in five plants per accession in the second leaves of control and stressed plants at time 9 and 12 daww Antioxidant activity was measured in the leaves using the Ferric Reducing Ability of Plasma (FRAP) assay according to (Rosales *et al.*, 2006). The FRAP assay was performed with FRAP reagent, i.e. 1 mmol L^{-1} 2, 4, 6-tripyridyl-2-triazine (TPTZ) and 20 mmol L^{-1} ferric chloride in 0.25 molL^{-1} sodium acetate (pH 3.6). An aliquot of 50 µl of leaf extract (10 mg per mL^{-1} in methanol) was added to 1 ml of FRAP reagent and mixed thoroughly. After the mixture had been left at ambient temperature (20 ºC) for 5 min, the absorbance at 593 nm was measured. Calibration was against a calibration curve (25–1600 molL^{-1} ferrous ion) constructed using freshly prepared ammonium ferrous sulphate.

Statistical analysis

All experiments were designed in a randomized complete block design. For ease of understanding, means of raw percentage data are presented in tables and figures. However, for statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = 180/π x arcsin[√(%/100)]) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 7th Edition, after which residual plots were inspected to confirm data conformed to normality. In addition Shapiro-Wilk test and Bartlett's test were performed to test normality and homogeneity of variances respectively. Significance of differences between means was determined by contrast analysis (Scheffe's). In addition least significant difference (LSD) values were added to tables and figures for comparison between each two accessions. When appropriate, tukey test ($p<0.05$) were used for multiple comparison among accessions.

For multivariate analysis the data were first analyzed using principal components analysis (PCA; Causton, 1987) according to the NIPALS algorithm (Wold, 1966). Briefly, this involves projecting a ('X') matrix formed from set of (N) data onto multidimensional space. Principal components (PCs) are linear combinations of original variables (known as loadings) which are used in the projection of the X matrix. Individual PCs are ranked (PC1, PC2, etc.) on the basis of the variance within the original dataset that is explained. PCA is an unsupervised method where no *a priori* knowledge of experimental structure is given. Thus, if there is clustering of either 2D or 3D projections of PCA from

replicate data, this indicates that the original experimental parameters are the sources of maximal variation. PCA was followed by discriminant function analysis (DFA) which is a supervised projection method (Manly, 1994). DFA then discriminated between groups on the basis of the retained PCs and the a priori knowledge of which values were replicates (either biological or machine). DFA was programmed to maximize the Fisher ratio (i.e. the within-class to between-class variance) and the similarity between different classes reflects the optimal number of PCs that are fed into the DFA algorithm. All calculations were performed in Pychem 2.0 (Jarvis *et al.*, 2006).

PC-DFA models were created to identify the variables or parameters associated with the differences between the susceptible and resistant accessions. The approach was based on deriving robust models where differences between classes were separated along a particular PC-DF axis. This allowed plotting the contributions of individual variables measured to the model ("loading vectors"). Those parameters that appeared > ± 1 standard deviation (STD) from the mean value loading were recorded as 'discriminatory' variables associated with the differences.

Due to the complex experimental structure and the need to derive PC-DFA models where classes were separated along particular PC-DF axes, differing approaches were followed, each generating a list of key parameters. Firstly PC-DFA plots were constructed for each individual time point (6, 9, 12, 15 and 18 daww). Since none of the models discriminate well between accessions, PC-DFA plots were constructed grouping different time points. In addition models were derived by grouping the accessions (i.e. most resistant vs. most susceptible or moderately resistant vs. themselves). This identified the key time points and parameters to discriminate susceptible and resistant accessions.

RESULTS

Screening for drought resistance sources

Following daily visual assessment of drought symptoms, AUDPC values were calculated for the 174 *Avena* accessions which could be grouped into 7 classes overall conforming to a normal distribution (Fig. 1A). Accessions from the most frequent class, with AUDPC values of between 31 and 35, were considered neither resistant nor susceptible to drought stress. Accessions with values lower than 20 were considered to be highly resistant to drought, with others with scores between 21 and 31 considered to be resistant or moderately resistant. Accessions with AUDPC values higher than 46 were considered to be highly susceptible but those between 35 - 45 exhibit moderate susceptibility. Thus, 31.6% of the accessions were regarded as moderately resistant, and 0.5% as highly resistant (Fig. 1A) with these classes including 44 *A. sativa* and 4 *A. byzantina* landraces and 8 commercial cultivars. Fig 1B represents the drought progress curves along the drought time course of Patones and Flega, the most resistant and susceptible accessions respectively. For all accessions the drought progress curve was a sigmoidal which in susceptible accessions scores rapidly (by ~1-2 days) started to increase and reached 5 by day sixteen. In contrast, the most resistant accessions maintained a score of 0 for at least 2-3 days and did not achieve a score of 5 score until at least 18 days after the imposition of drought stress. For further analysis of drought resistance components 14 accessions were selected according to their AUDPC values, including 1 highly resistant (Patones), 1 resistant (Gen16), 9 moderately resistant (Mirabel, Anchuela, Gen17, Gen76, Gen100, Gen122, Gen124, Gen125 and Gen 135), 1 moderately susceptible (Rapidena) and 2 highly susceptible (Flega and Alcudia) accessions. Statistical comparison of the specific AUDPC values of these accessions showed that the highly susceptible accessions significantly differed from the moderately resistant, resistant and highly resistant ones (Tukey $p<0.05$: Table 1).

Field assessment of yield of selected accessions grew in water prone conditions showed a high correlation with the water stress tolerance showed under the assayed controlled conditions (Online Resource 2).

Characterisation of resistance mechanisms underlying responses to drought

Assessment of RWC (Fig. 2a) showed values of approximately 90% in all accessions before withholding water with no significant differences amongst accessions. Following the imposition of drought conditions there was a decline in RWC in all accessions although those accessions classified as highly susceptible (i.e. Alcudia and Flega) exhibited a more rapid decline compared to the most resistant ones (i.e. Patones and Gen124). Overall statistical analysis showed significant differences among accessions ($p < 0.001$), time points after withholding water ($p < 0.001$) and interaction between these factors ($p < 0.001$). Interestingly, accessions such as Gen125, Anchuela and Gen17 considered moderately resistant rapidly reduced their RWC and at day 15 and 18 exhibited no significant difference to Alcudia and Flega (Fig. 2a).

A similar pattern was observed following assessment of CMS (Fig. 2b). Thus, overall analysis showed significant differences among accessions ($p < 0.001$), time points ($p < 0.001$) and interaction between these factors ($p < 0.001$). CMS values ranged from 100 in non-stressed plants to 20% in the most susceptible accessions after 18 days of drought treatment. This reduction in CMS with time was observed in all accessions. However, we did not find a significant linear correlation between RWC and CMS values. This suggested that not all accessions maintained membrane integrity under drought stress in the same way. To further investigate this behaviour we derived model curves based on observed values. RWC and CMS values fitted with negative gompertz curves (non-symmetrical) in all accessions, except Gen100 and Gen125 for RWC and Patones for CMS that fitted with negative logistic type curves ($p<0.001$). Fig 2c, d shows the observed points and derived curves for the most susceptible, Flega, and most resistant accession, Patones. Table 2 shows the point of inflexion, and the lower and upper asymptotes (floor and ceiling) for all accessions for RWC and CMS. Regarding to RWC, susceptible accessions such as Flega showed the earlier inflexion point and also the lowest floor together with Alcudia. By contrary Patones and also Gen135 showed inflexion points at longer times and high floor values. All accessions shower similar ceiling values of approximately 85% of RWC (Table 2). Regarding CMS, Gen 122, Gen125 and Gen135 showed the earliest inflexion points but not the lowest floor values that were shown by Flega, Alcudia, Gen 100 and Gen 17. Interestingly Patones showed a later inflexion point together with a high floor value (Table 2). CMS values at a limit RWC of 45% calculated from curves showed that Patones had the higher CMS. However other accessions, such as Gen16 and Gen124, considered moderately resistant exhibited greater membrane damage (Table2).

Stomatal conductance

In most accessions measurements taken two hours after the onset of the light period and the in the middle of the light period tended to be higher than those taken two hours before the end of the light period (Fig. 3A). Water-stressed plants showed a rapid reduction in g_l with respect to the controls, especially in susceptible accessions such as Flega and Alcudia (at ~ 134 h after withholding water). Interestingly, other accessions considered moderately susceptible and resistant such as Rapidena and Anchuela respectively also showed this trend (between 110-158 h after withholding water) (Fig. 3A). In order to compare the patterns of g_l between accessions we calculated the difference between the area under the conductance progress curve (AUCPC) of control and stressed plants. The values depicted in Table 3 shows that Flega, Alcudia (both classed as susceptible), Rapidena (moderately susceptible) and Anchuela (moderately resistant) exhibited a reduction in AUCPC values which were significantly greater than that observed in the most resistant accessions such as Patones ($p < 0.001$). However, other accessions such as Gen76 and Gen100 considered

moderately resistant also showed significant differences with Patones ($p<0.001$) (Fig. 3B). Such observations indicated mechanisms of drought resistance amongst the germplasm collection that were not only associated with stomatal regulation.

Assessing infrared temperature

Analysis of infrared (IR) temperatures of control plants indicated significant differences between accessions ($p <0.001$), sampling times ($p <0.001$) and interaction between these factors ($p <0.001$). This indicated that the accessions were at very different physiological statuses even at optimal environmental conditions. For example, accessions such as Flega, Gen76 and Anchuela had the lowest temperature values in most of the sampling times assessed whereas Gen124 had the highest values (data not shown). Interestingly, water stressed plants exhibited a dramatic increase in leaf temperature with time after withholding water when compared to controls ($p<0.001$) with highly significant differences between accessions ($p<0.001$; Fig. 4A). The overall mean increase in temperature in water stressed plants was 1.73 ºC but was more dramatic at later time points. Thus, whilst between T_6 and T_{12} the mean increase in temperature of water stressed plants respect to the controls was by 1.27 ºC between T_{12} and T_{18} this was 3.45 ºC. However not all accessions responded in the same manner to the drought stress during the time course as indicated the interaction between these two factors ($p<0.001$). At T_6 Gen122, Gen135 and Rapidena showed the lowest differences with respect to their controls whilst Gen17 showed the highest increase ($p<0.001$). At T_{12}, Patones and Rapidena showed the lowest temperature increase but Anchuela, Gen 122, Mirabel, Alcudia and Flega had the highest increases ($p<0.001$) compared to controls. At T_{18} Patones showed the lowest temperature increase of any of the accessions (Fig. 4A). Fig. 4B shows the infrared pictures of controls and drought stressed Flega and Patones plants at T_{15} and indicates the higher increase in temperature with respect to the control observed in the susceptible Flega compared to that in Patones.

Water use efficiency

WUE in terms of dry biomass per litre of water consumed ranged between 1.55 (Gen 16) and 2.63 (Mirabel) respectively (Table 3). Data showed differences among accessions with Mirabel and Gen 17 showing significant higher values compared to the other accessions.

Lipid peroxidation and lipoxygenase activity

All accessions exhibited significantly greater MDA content at both, 9 and 12 d. a .w. w ($p< 0.001$) compared with their controls. However, differences amongst accessions were highly significant ($p<0.001$) as were those between time points ($p=0.022$) with lower relative MDA content 9 than 12 daww (Fig 6A). We also observed significant accession x time points ($p =0.004$) and accession x treatment ($p =0.005$) interactions. Thus, at 9 daww highly resistant accessions such as Patones, Mirabel, and Gen122 had the lower MDA content whereas the most susceptible or moderately susceptible accessions such as Flega and Rapidena had the highest levels (Fig. 5A). Nevertheless, other accessions such as Alcudia which were considered highly susceptible did not significant differ in MDA content when compared with resistant Patones or Mirabel. In the same way resistant accessions such as Gen16 and Gen17 had similar concentrations to that found in the susceptible Flega.

Analysis of lipoxygenase (LOX) activity in non-stressed plants showed no significant differences among accessions. However, we observed a significant increase in LOX activity in water stressed plants respect to their controls, which differed amongst accessions ($p<0.001$) and time points ($p<0.001$) which at 9 daww was lower than that at 12 daww (Fig. 5B). The lack of interaction accession x time point indicated that all accessions increase LOX activity with time after withholding

water in a similar manner. We observed very high levels of LOX activity in the highly susceptible and moderately susceptible accessions Flega, Alcudia and Rapidena, but also in Anchuela considered moderately resistant (Fig. 5B). The LOX activity of the other accessions did not significantly differed to that from Patones except Gen125 at 12 daww. There was an overall correlation between LOX activity and MDA content ($r= 0.856$; $p<0.001$) but not between genotypic LOX activity and MDA content. Thus, we found a significant correlation between LOX and lipid peroxidation in Alcudia ($r=0.874$; $p=0.018$), Anchuela ($r=0.847$; $p<0.001$), Flega ($r=0.904$; $p=0.021$), Gen122 ($r=0.850$; $p=0.002$), Gen125 ($r=0.864$; $p=0.03$) and Gen17 ($r=0.834$; $p<0.001$) but not in the rest of accessions.

Leaf chlorophyll content

Analysis of SPAD chlorophyll meter reading (SCMR) showed significant differences between accessions ($p <0.001$), treatment ($p <0.001$) and time points ($p<0.001$) and interaction between all factors ($p <0.001$). This indicated that accessions respond very differently to water stress along the course of the drought experiment. Thus, Patones, Mirabel, Gen135 and Gen122 had a very low decrease in chlorophyll following the imposition of water stress compared to their controls, whilst accessions such as Flega, Rapidena, Alcudia, Gen17 and Gen124, showed a rapid decrease ($p<0.001$) (Fig. 6). The highest overall differences between accessions respect to SCMR were observed from 15 days after withholding water. However, significant genotypic differences were observed at each time point after withholding water (p between 0.01 and 0.001). Interestingly when we assessed the SCMR in controls plants we also observed differences between accessions ($p<0.001$), time points ($p<0.001$) and interaction between these factors. Differences among control plants were observed at all timepoints, with Mirabel and Patones showing the highest SCMR in 6, 9, 12 and 15 days and Flega, Gen122 and Gen125 showing the lowest values in most time points assessed.

Antioxidant activity

Antioxidant activity significantly increased in leaves of water stressed plants compared to their controls ($p<0.001$) with overall means of 49.7 and 75.5 μmol g^{-1} fresh weight for control and drought treated plants respectively. However, overall there were no significant differences amongst accessions, time points or interactions between these factors. Only when analyzed separately, were significant increases detected in Gen124 at 12 daww ($p=0.04$) with respect the other accessions except Gen 16, Gen122 and Gen125 (Fig. 7).

Selecting key physiological traits linked to drought resistance using multivariate statistical approaches.

Following the assessment of the above mentioned physiological traits during drought stress, a multivariate analysis was performed in order to determine those traits that better discriminate between susceptible and resistant accessions. In total 28 variables were studied derived from the data already presented. Unsupervised Principal Component Analysis (PCA) the whole dataset including all variables could not separate between accessions; hence, supervised PC-Discriminant Function Analysis (PC-DFA) was performed on 19 PCs (explaining 99% of the total variation) but this still failed to discriminate between accessions. To simplify the analyses, those variables from most extreme sampling times were removed; i.e. 6 daww (since at this time water stress was very low and most accessions had not registered any physiological change) and 18 daww (where most accessions exhibited the most extreme damage). PC-DFA plots (Fig. 8A) showed that it was now possible to distinguish between the highly resistant, the resistant and the susceptible accessions. PC-DFA indicated that leaf temperature at 12 and 15 daww, (based on PC loading vectors) following by AUDPC, and RWC at day 15 were the individual parameters that contributed most to the derived projections (Online Resource 3A). In addition, hierarchical cluster analysis (HCA) based on the PCs separated the most susceptible accessions, Flega and Alcudia and also the most resistant accession;

Patones, from other resistant and moderately resistant accessions (Fig. 8B). The model did not discriminate between the considered moderately susceptible accession, Rapidena, and the moderately resistant accessions (Fig. 8A, B).

PCAs and DFAs projections of the trait data showed a very different trend depending on the sampling time assessed (Fig. 9). When analyzed those parameters recorded at 6 and 9 daww none of the PC-DF axes explained differences among any of the accessions (Fig. 9). By 12 daww it was possible to discriminate between the most susceptible accessions, Flega and Alcudia, from other accessions with examination of the PC loading vectors indicating that infrared temperature was the parameter that most explained this variation following by AUCPC and LOX (Online Resource3B). Analysis of parameters recorded at 15 daww allowed the discrimination between the most resistant, Patones, and the remaining accessions with again infrared temperature and also AUCPC as the main loading vectors explaining this difference (Fig. 9), (Online Resource 3C). A similar trend could be observed when traits taken at 18 daww were analyzed, with infrared temperature and SCMR measurements as main parameters explaining the model (Online Resource 3D).

Since the parameters that most contributed to the differences among accessions in the general model (Fig. 8A), were leaf temperature at 12 and 15 daww, AUDPC and RWC at T_{15} these were also analysed together and separately from the others variables (Fig. 10). In these analyses PCA results differed greatly from the general model (Fig. 8) and showed good discrimination among accessions. In addition, when focussed only on these parameters, DFA analysis could also discriminate between the highly resistant, the moderately resistant and the highly susceptible accessions (Fig. 10).

Since from the breeding point of view it would be highly desirable selection of resistant accessions in the absence of the stress, those parameters from which genotypic differences were found among control plants were also analysed. However, in this analysis, neither PCA or DFA allowed discrimination among accessions (Fig. 11). We also compared several of the accessions in order to determine other key traits discriminating between them (Online Resource 4, 5). PCA and DFA models clearly discriminated between the most susceptible, Flega, and most resistant, Patones, accession when analyzed separately from the rest (Online Resource 4). SCMR9, AUCPC, LOX12, RWC12, IR12, IR15, SCMR15 and LP12 were the parameters that most contributed to the discrimination between these two accessions. When comparing only the moderately or resistant accessions three different clusters were observed, the first one including Gen135, Gen122, Gen124, Gen125, Gen100 and Gen17, the second one with Gen16, Gen 76 and Rapidena, and the third one with Mirabel, and Anchuela (Online Resource 4). The significant parameters explaining this grouping were leaf temperature at 12, 15 and 18 daww, SCMR at 18 daww and LOX at 9 daww.

DISCUSSION

Drought is currently one of the main constrains preventing crops plants from expressing their full genetic potential. The identification of sources of drought tolerance in germplasm which is sexually compatible with elite crops is crucial to secure productivity. Thus, we sought to find novel sources of tolerance to drought in an oat germplasm collection consisting of 174 accessions. Following a visual assessment of the oat collection during a time course of withholding water, 11 accessions of *A. sativa* and *A. byzantina* including landraces and commercial cultivars were identified as being either highly resistant, resistant or moderately resistant to drought. *Avena sativa* and *A. byzantina,* sometimes known respectively as the white and red oats, are the main cultivated oats. They are self-pollinating hexaploids and sexually compatible with hybridizing techniques (Stevens *et al.*, 2004) so that our identified germplasm could be readily introduced into breeding programmes. Crucially, field assessment of yield under water prone conditions correlated with the drought

tolerance observed under controlled conditions thereby indicating the usefulness of the assay in young plants. Small differences between field and controlled conditions might be due to other drought resistance mechanisms expressed in field and/or older plants such as specific avoidance mechanisms (root architecture, date of flowering etc) which were not assessed in this work. In addition experimental factors such as pot size for controlled experiments and/or row spacing for field experiments could influence somewhat the observed differences.

Further we characterize the selected accessions assessing several physiological drought tolerance mechanisms and used a multivariate approach in order to determine the key features/responses explaining the drought tolerance and susceptibility in oat. This is of high practical importance for breeding since the lack of effective selection criteria is considered to be a major impediment to breeding for drought-prone environments (Araus *et al.*, 2002; Ouk *et al.*, 2006; Venuprasad *et al.*, 2007). Many drought associated markers have been extensively studied by many authors. However, many are based solely on correlation – i.e. they simply occur at the same time as drought stress. However, the causal relationship to drought stress tolerance is often not fully assessed. In line with this, we observed that several of the traits usually associated with drought resistance were present in many, but not in all, of the resistant accessions. Indeed, even susceptible accessions possessed some features associated with drought resistance. Such observations, ably demonstrated how difficult it has been to best discriminate drought tolerance within a group of oat accessions.

Among previously reported drought-linked physiological features, we assessed several associated to water relations such as RWC, g_1, leaf temperature and WUE. RWC is an appropriate measure of plant water status in terms of the physiological consequence of cellular water deficit. Other parameters such as water potential is useful in dealing with water transport in the soil-plant-atmosphere continuum, but does not account for osmotic adjustment (OA) which is a powerful mechanism of conserving cellular hydration (Islam *et al.*, 2011). In contrast, RWC takes into account the possible effect of both leaf water potential and OA (Islam *et al.*, 2011; Blum, 1999). Predictably, RWC declined in all accessions after water was withheld but the rates of RWC reduction differed among accessions. Susceptible accessions rapidly reduced their RWC but some of the moderately resistant accessions were not very different in their RWC loss rates. In these latter accessions specific mechanisms such as the preservation of cell membrane integrity might allow the maintenance of metabolic activity even at low RWC. Indeed, we observed that most of these had high values of CMS at low (45%) RWC. Cell membrane is one of the main cellular targets to different stresses (Levitt, 1980) and maintenance of membrane stability during drought is important for normal physiological metabolism to continue under low water potential (Tripathy *et al.*, 2000). However, phenotype selection only for CMS may not always be accurate for breeding purposes because of its complex nature and its strong interaction with the environment (Tripathy *et al.*, 2000). Ideally, it is important to evaluate this trait under controlled environment and equal RWC for accurate comparisons as demonstrated in this study and these are not readily transferred to a field situation.

In addition to RWC, g_1 is an important component contributing water relations during drought stress. Stomata close progressively as drought progresses, followed by parallel decreases of net photosynthesis. Indeed, stomatal closure has been suggested as the main determinant for decreased photosynthesis under mild to moderate drought (Cornic and Massacci, 1996; Medrano *et al.*, 2002), although some authors disagree (Tezara *et al.*, 1999). We observed a reduction in g_s in all assessed accessions during the water stress experiment although differences in stomatal conductance of stressed and controls plants differed greatly among accessions. As previously reported (Medrano *et al.*, 2002), stomata of most accessions closed in response to drought before any change in leaf water content was detectable. This is attributed to the abscisic acid (ABA) root-to-leaf signalling promoted as the soil dries. Thus, g_s is responsive to several external (soil water availability, vapour pressure deficit) and internal (ABA, leaf water status) factors related to drought

and can be considered as an integrative parameter reflecting photosynthetic response during water stress (Medrano *et al.*, 2002). Accordingly, accessions such as Alcudia, Flega, Anchuela and Rapidena with the highest decreases in stomatal conductance compared to their controls during the water stress (AUCPC values) would be photosynthetically more affected than Patones, Gen16, Gen17 Gen122, Gen124 and Gen135. Stomatal movements are very dynamic due to complex regulation stated above. For this reason three measurements during the light time course were performed. This was preferred to solely midday g_s readings because, as drought becomes progressively intense, the daily peak conductance is displaced (Flexas *et al.*, 2000) and only one measurement might not display accurate g_s curves.

When water evaporates from the surface of the leaf, it becomes cooler due to stomatal conductance (beside vapour pressure deficit). Thus, leaf temperature and temperature depression compared to ambient air temperature is a good indicator of a genotypes' physiological fitness (Araus *et al.*, 2002). Since measurements performed in this study were achieved under controlled environmental conditions, differences in leaf temperature among accessions could be easily compared in stressed and non-stressed plants. Most moderately resistant accessions had very low increases of leaf temperature 12 d. a .w. w. suggesting good physiological homeostasis since cooling improves the photosynthetic activity and prevents premature senescence. Clearly, such homeostatic mechanisms are compromised in most accessions by 18 daww..

Regarding WUE, although it is one of the most studied parameter related to drought resistance, there is a constant debate of 'putative' drought resistance mechanisms, 'water-use efficiency', and their interrelationship and associations with yield potential. WUE for yield is often equated in a simplistic manner with drought resistance. However, several authors (i.e (Blum, 2005; Condon *et al.*, 2002) suggested that selection for higher WUE assuming that it equated with improved drought resistance or improved yield under stress may in fact lead to the selection of negatively acting factors. Thus, genotypic variations in WUE are mainly driven by variations in water use rather than by variations in plant production or assimilation per given amount of water use. In line with the suggestions of such as Blum (2005) our data did not show a clear correlation of WUE with drought tolerance suggesting that WUE did not highly contribute to the observed genotypic variation to drought.

The above reported mechanisms directed to cell/tissue water conservation are tightly linked to physiological traits associated with oxidative damage and/or activation of antioxidant mechanisms. Under drought, stomata close and this limits CO_2 fixation in the chloroplast so that electron flow in the light reactions exceeds that required for CO_2 assimilation. This leads to the over-reduction of photosynthetic components and the resulting production of reactive oxygen species (ROS), such as superoxide (O_2^-), hydrogen peroxide (H_2O_2), or the hydroxyl radical (OH^-) (Cruz *et al.*, 2004). These ROS, if not quenched by antioxidant machinery, can seriously disrupt metabolism through membrane lipid peroxidation, chlorophyll loss and protein carbonylation (Basu *et al.*, 2010). During the lipid peroxidation, protons are abstracted from for example, phospholipids to initiate a lipid radical ($L^{\cdot}$)–lipid hydroperoxide (LOOH) chain reaction ($LH + {}^{\cdot}OH \rightarrow L^{\cdot\cdot} + H_2O$; $L^{\cdot\cdot} + O_2 \rightarrow LOO^{\cdot}$; $LOO^{\cdot} + LH. \rightarrow LOOH + L^{\cdot}$ etc. in a propagative cycle). The peroxidation of non-saturated groups within acyl chains [also known as polyunsaturated fatty acids (PUFA)] in a membrane would severely disrupt its integrity. Our results shows an increase in the PUFA breakdown product MDA content, in all accessions with time after withholding water with higher increases in susceptible accessions. However, several of the moderately resistant accessions had similar MDA content to the susceptible ones. Further, in several of the accessions we found a correlation between the MDA content and the lipoxygenase activity. This could indicate that in these accessions most of the peroxidation of the membrane polyunsaturated fatty acids was mediated by this enzymatic reaction rather than free radical chemistry. Furthermore, in several of the accessions with high MDA content we also observed considerable chlorophyll-loss in stressed compared to control plants as indicated by SCMR values

(Yadava, 1986) which can also suggests transpiration efficiency (Nageswara-Rao *et al.*, 2001). Our results showed that several of the resistant accessions maintained chlorophyll levels upon drought stress indicating the maintenance of photosynthetic related activities. Interestingly, we also observed significant differences among controls in all time points. Control plants of accessions showing low SCMR in most time points assessed might indicate an early senescence of the leaves, than in the case of i.e. Flega would add to the chlorophyll loss due to drought stress. Oxidative damage in the plant tissue above reported may be alleviated by a concerted action of both enzymatic and non-enzymatic antioxidant mechanisms. These mechanisms include scavenge of radicals by β-carotenoids, α-tocopherol, ascorbate, glutathione, anthocyanins, flavonoids, carotenoids (Mittler and Blumwald, 2010), and enzymes including superoxide dismutase, catalase, glutathione reductase, and ascorbate peroxidase (Asada, 1992; Halliwell, 1987). The total non-enzymatic antioxidant activity estimated by the FRAP assay revealed no differences amongst accessions in the present study either at 9 or at 12 daww. This does not rule out the possibility of significant differences between accessions in specific non-enzymatic antioxidants or antioxidant enzymes which should be studied in detail. However, antioxidant activity was not further assessed in the present study since it is more difficult to test in a breeding population in a realistic manner.

Considered individually, it is difficult to determine which of the above stated drought linked physiological features is the crucial one/s responsible for the tolerance phenotype in oat. However, by screening large germplasm pools and following PC-DFA models based on all these variables assessed together, we identified specific water use related features such as leaf temperature and RWC as the main traits indicative of drought tolerance in oat. Other physiological processes involved in cell/tissue water maintenance including g_1 and those reflecting oxidative damage and antioxidant defence albeit linked with the resistance responses might be considered as weakly correlated events not suitable for discriminating among oat accessions.

Interestingly not all measurement of leaf temperature and RWC discriminated among accessions but only the intermediates taken at time 12 and 15 daww together with the AUDPC assessed during the whole time course of water stress. With selected variables, both the unsupervised PCA and the DFA analyses were useful in discriminating between the most susceptible, the moderate and most resistant accessions indicating its appropriateness for breeding selection. Furthermore, the very different nature of the plant material assessed; landraces, and commercial cultivars from *A. sativa* and *A. byzantina* species point to the strength of this approach to be used in populations with very different genetic backgrounds. The study also show that the physiological status of the control plants is not enough to discriminate among accessions and parameters for selection need to be also measured under the drought stress in order to perform an accurate selection.

Overall, this work allowed for the first time the ranking of many supposed drought resistance traits in order of degree of importance within oat, highlighting those with a causal relationship to drought stress tolerance and not only correlated with it in determinate accessions. Our approach was to encompass as much oat biodiversity as possible but screened under controlled conditions. Screening under natural stress conditions is difficult because of the irregular and erratic drought response (Venuprasad *et al.*, 2007) whereas controlled conditions allowed the inexpensive and robust screening of large populations with optimized protocols for selection of plants carrying specific physiological mechanisms that can be coupled later with yield assessments in the field for selected accessions. As such, this initial stage can be readily adopted by crop breeders. By combining information on the basis of yield limitation under contrasting environments with the new physiological/ biochemical /molecular selection tools, the probability of accelerating the rate of genetic progress through plant breeding will be significantly increased (Araus *et al.*, 2002). This study shows the potential of multivariate analysis as robust approach to target key mechanisms

responsible for drought tolerance in oat and can be used to speed genotype selection from large breeding populations otherwise difficult and expensive to test.

ACKNOWLEDGMENTS

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Supplementary material.

Additional Supplementary material may be found in the online version of this article:

Online Resource 1. Visual assessment of drought symptoms during a 19 days time course of drought.
Online Resource 2. Yield of selected accessions in prone water conditions.
Online Resource 3. PC-DFA loading vectors contributing to the derived projections that discriminated between accessions
Online Resource 4. Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the most susceptible (Flega) and Most resistant (Patones) accessions based on all variables assessed
Online Resource 5. Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the moderately resistant accessions based on all variables assessed.

Tables

Table 1: Area Under Drought Progression Curve (AUDPC) values of selected accessions.

Accession	Species	AUDPC	Test Tukey (p<0,05)	Preliminary Classification
Alcudia	*A. sativa*	46.3±0.39	c*	Highly Susceptible
Anchuela	*A. sativa*	30.4±2.46	ab	Moderately Resistant
Flega	*A. sativa*	49.2±0.84	c	Highly Susceptible
Mirabel	*A. sativa*	28.7±2.13	ab	Moderately Resistant
Patones	*A. sativa*	19.0±1.18	a	Highly Resistant
Rapidena	*A. sativa*	40.9±1.75	bc	Moderately Susceptible
Gen16	*A. sativa*	21.1±3.77	a	Resistant
Gen17	*A. sativa*	27.6±2.97	ab	Moderately Resistant
Gen76	*A. byzantina*	30.1±2.01	ab	Moderately Resistant
Gen100	*A. byzantina*	30.6±0.98	ab	Moderately Resistant
Gen122	*A. sativa*	27.5±5.11	ab	Moderately Resistant
Gen124	*A. sativa*	29.5±4.54	ab	Moderately Resistant
Gen125	*A. byzantina*	30.7±2.56	ab	Moderately Resistant
Gen135	*A. byzantina*	29.62±1.88	ab	Moderately Resistant
L.S.D. (5%)		8.723		

*Different letters indicates significant differences according to Tukey test (p<0.05) among accessions

Table 2. Model derived parameters of Relative Water Content and Cell Membrane Stability curves and CMS values derived from the models at 45% of relative water content.

Accession	Relative Water Content (%)			Cell Membrane Stability (%)			
	Inflexion	Floor	Ceiling	Inflexion	Floor	Ceiling	CMS at 45% of RWC
Alcudia	14,6±0,2	18,9±2,9	87,4±3,4	14,8±0,5	17,9±7,4	103,4±9,4	53,86
Anchuela	14,1±0,3	26,8±2,9	85,6±3,3	14,9±0,3	26,7±4,6	100,3±5,6	61,26
Flega	11,5±0,4	19,54±2,0	87,2±4,4	14,3±0,5	17,5±6,8	106,3±9,3	59,98
Mirabel	14,2±0,3	35,1±2,8	88,8±3,3	14,4±0,4	40,1±4,5	101,8±5,7	52,53
Patones	15,7±0,5	36,1±4,3	87,5±4,8	15,5±0,6	40,4±1,4	99,5±24,0	66,97
Rapidena	14,0±0,3	25,3±3,2	88,3±3,6	14,0±0,3	24,2±4,1	101,8±5,0	48,92
Gen16	14,6±0,8	30,9±6,9	87,8±8,0	14,1±0,4	32,6±4,7	108,1±5,9	43,75
Gen17	14,7±0,4	29,1±3,8	89,4±4,3	15,1±0,9	11,4±16,4	114,4±19,6	47,29
Gen 76	14,9±1,5	43,0±3,5	82,1±3,8	14,5±0,3	47,5±4,6	103,1±5,1	50,76
Gen 100	17,0±1,4	29,7±3,5	89,1±3,8	17,0±0,7	14,6±3,8	106,7±5,5	47,19
Gen122	12,2±1,1	36,8±3,7	86,2±4,0	13,8±0,4	35,4±4,6	107,1±5,5	58,56
Gen124	15,6±0,7	39,5±4,2	85,4±4,8	14,1±0,4	40,7±5,0	105,8±6,1	42,51
Gen 125	22,3±0,3	41,7±3,4	87,9±3,9	12,1±0,3	50,1±3,9	102,3±5,4	50,12
Gen135	12,1±0,8	31,8±1,8	84,8±2,5	13,1±0,5	42,8±4,5	108,7±5,9	62,46

Table 3: Area under the conductance progress curve values during the drought time course experiment and water use efficiency of the selected oat accessions.

Accession	AUCPC	WUE
Alcudia	3777±840,5	2,02±0.05
Anchuela	3773±1085,1	2,10±0.11
Flega	2546±903,1	1,95+0.04
Mirabel	1715±800,4	2,64±0.16
Patones	946±493,5	1,73±0.06
Rapidena	3085±1071,3	1,84±0.05
Gen16	895±540,1	1,55±0.09
Gen17	912±688,1	2,49±0.13
Gen76	2422±611,4	1,62±0.11
Gen100	2218±739,2	1,59±0.08
Gen122	1022±424,9	1,70±0.12
Gen124	371±309,4	1,84±0.12
Gen125	1889±551,1	1,77±0.07
Gen135	629,9875±185,5	1,78±0.13

Figure Legends

Fig.1. Visual assessment of drought symptoms during a 19 days time course of drought **A.** Classification of *A. sativa* (■) and *A. byzantina* (□) wild accessions and in commercial oat cultivars (□) plants according to the Area Under the Drought Progression Curve (AUDPC) based on a 5-0 visual scale of drought damages where 0= completely healthy plant and 5= completely wilted plant. According to this, accessions were classified as highly susceptible, moderate susceptible, moderate resistant, resistant and highly resistant. **B**. AUDPC curves of the most susceptible, Flega (solid circles), and most resistant, Patones (open circles), accession. Data are based on five plants per accession distributed in randomized blocks + standard error.

Fig. 2. Relative Water Content and Cell Membrane Stability assessment. **A.** RWC and CMS of the selected oat accessions along a time course of drought. Data are mean of ten replicates per accession and treatment. L.S.D bar ($p<0.05$) is represented for accession comparison in any of the times points. Alcudia (◇); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□); Gen17 (▲); Gen76 (◆); Gen100 (○); Gen122 (◇); Gen124 (□); Gen125 (△); Gen135 (●). **B**. Original (solid line) and model derived (dotted line) of RWC and CMS curves of the most susceptible, Flega, and most resistant, Patones, accessions. Equation of the fitted curve and the correlation coeficient are also depicted in the figure.

Fig. 3. Stomatal conductance (mmol m-2s-1) of the selected oat accessions along a time course of drought. Three measurement were taken during 10 days: two hours after beginning of light period, in the middle, or two hours before the end of light period. Data are based on ten replicates per accession and treatment. Control are represented by open circles and drought treatment by solid triangles.

Fig. 4. Infrared Temperature assessment. **A.** Increase in Infrared Temperature (IRT) in ºC, in the selected oat accessions at day 6 (white bars) and day 12 (grey bars) and day 18 (black bars) after withholding water. Data are based on five plants per accession and treatment and four IR readings, each in one different leaf, per plant. **B.** Infrared temperature images of the most susceptible, Flega, and most resistant, Patones, accession, 12 days after withholding water.

Fig. 5. Lipid peroxidation of the selected oat accessions at day 9 (open bars) and day 12 (solid bars) after withholding water. **A.** Relative content of malondialdehyde in second leaves respect to its corresponding controls. Data are based on five replicates per accession and treatment $\pm$ standard error. **B**. Relative lipoxygenase activity in the second leaf respect to its corresponding controls. Data are based on five relicates per accession and treatment $\pm$ standard error.

Fig. 6. Decrease of the SPAD Chlorophyll Meter Readings (SCMR) respect to the control in the selected oat accessions. SCMR were assessed in the second leaf along a time course of drought. Data are based on five replicates per accession and treatment and three SCMR per leaf. L.S.D bar ($p<0.05$) is represented for accession comparison in any of the times points. Alcudia (◇); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△); Gen16 (□); Gen17 (▲); Gen76 (◆); Gen100 (○); Gen122 (◇); Gen124 (□); Gen125 (△); Gen135 (●).

Fig. 7. Test FRAP of antioxidant activity in the second leaf of the selected oat accessions at day 9 (open bars) and day 12 (solid bars) after withholding water. Activity is expressed in percent respect to its corresponding controls. Data are based on five replicates per accession and treatment $\pm$ standard error.

Fig. 8. Multivariate analysis of selected oat accessions according to the different parameters assessed **A.** Scatterplot of Discriminant Function Analysis scores of components 1 and 2 based on the different

parameters assessed (AUDPC, RWC, CMS, Stomatal Conductance, MDA, LOX, Antioxidants, SCMR, and IRT) at different time points after witholding water. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●). **B**. Hierarchical Cluster Analysis of the selected oat accessions according to the model represented in A.

Fig. 9. Multivariate analysis of selected oat accessions according to the different parameters assessed at different sampling times. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (●).

Fig. 10. Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 according to significant parameters obtained from the general model (AUDPC, RWC15, IRT12 and IRT15). Alcudia (▽); Anchuela (■ Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△; Gen135 (●

Fig. 11. Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 according to parameters obtained from control plants. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (●); Gen122 (▽Gen124 ( )□Gen125 ( ); △en135 ( ). ●

FIGURES

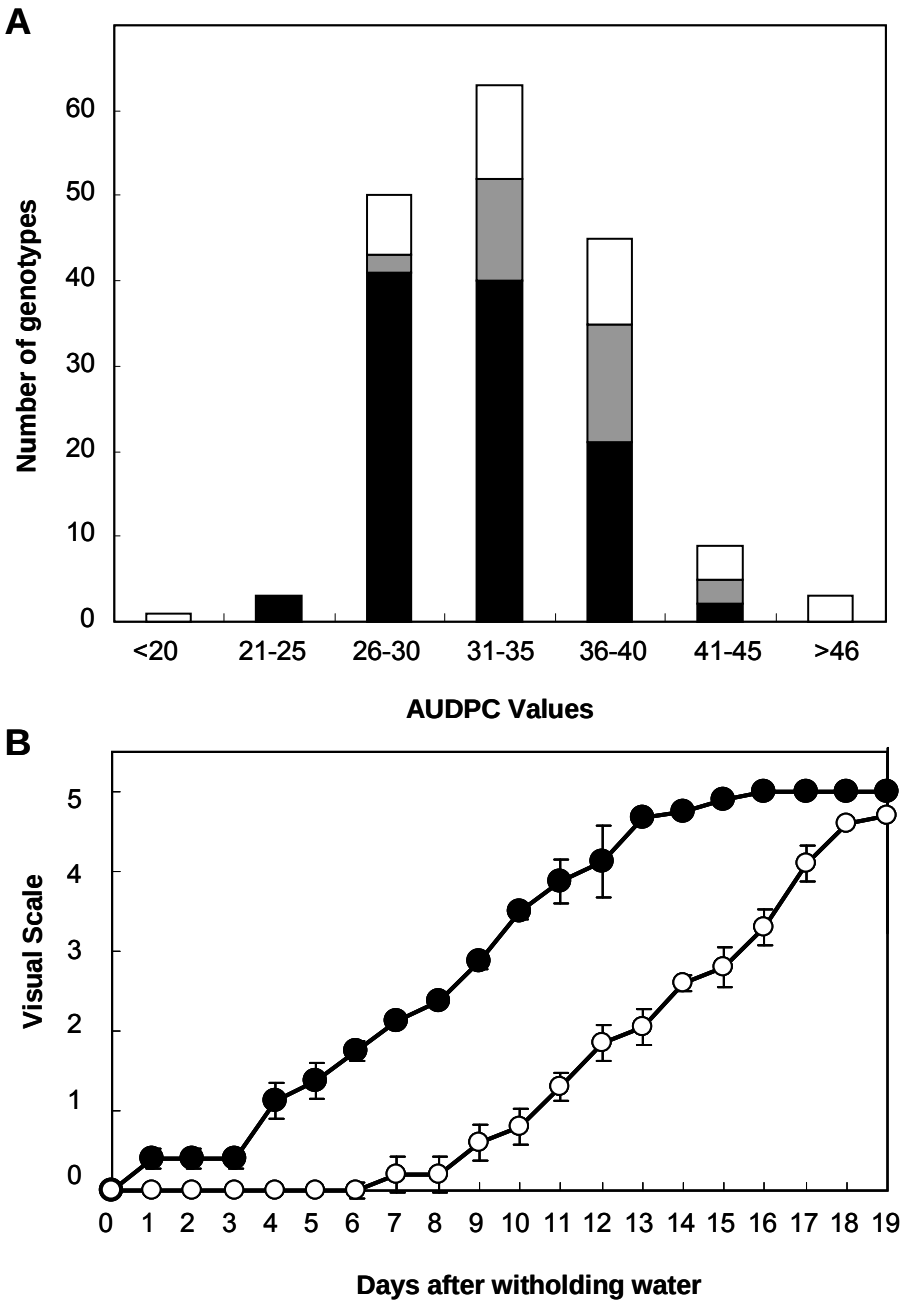


Figure 1.

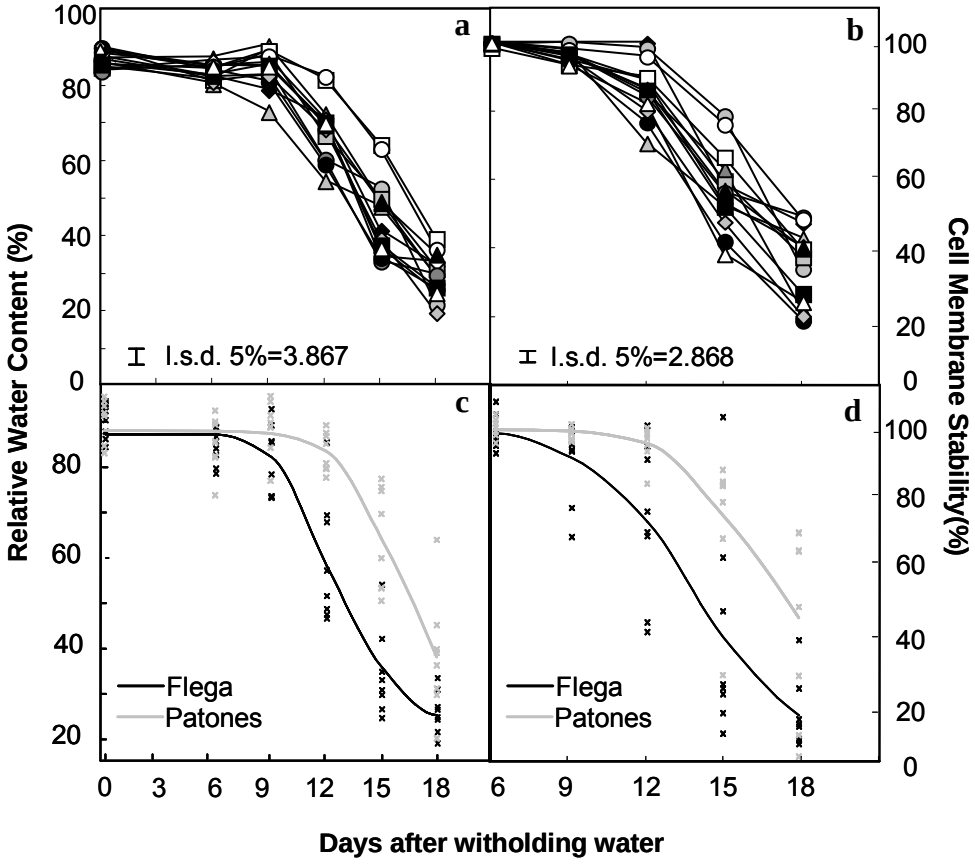

a
b
c
d
I l.s.d. 5%=3.867
I l.s.d. 5%=2.868
Relative Water Content (%)
Cell Membrane Stability(%)
Flega
Patones
Days after witholding water

Figure 2.

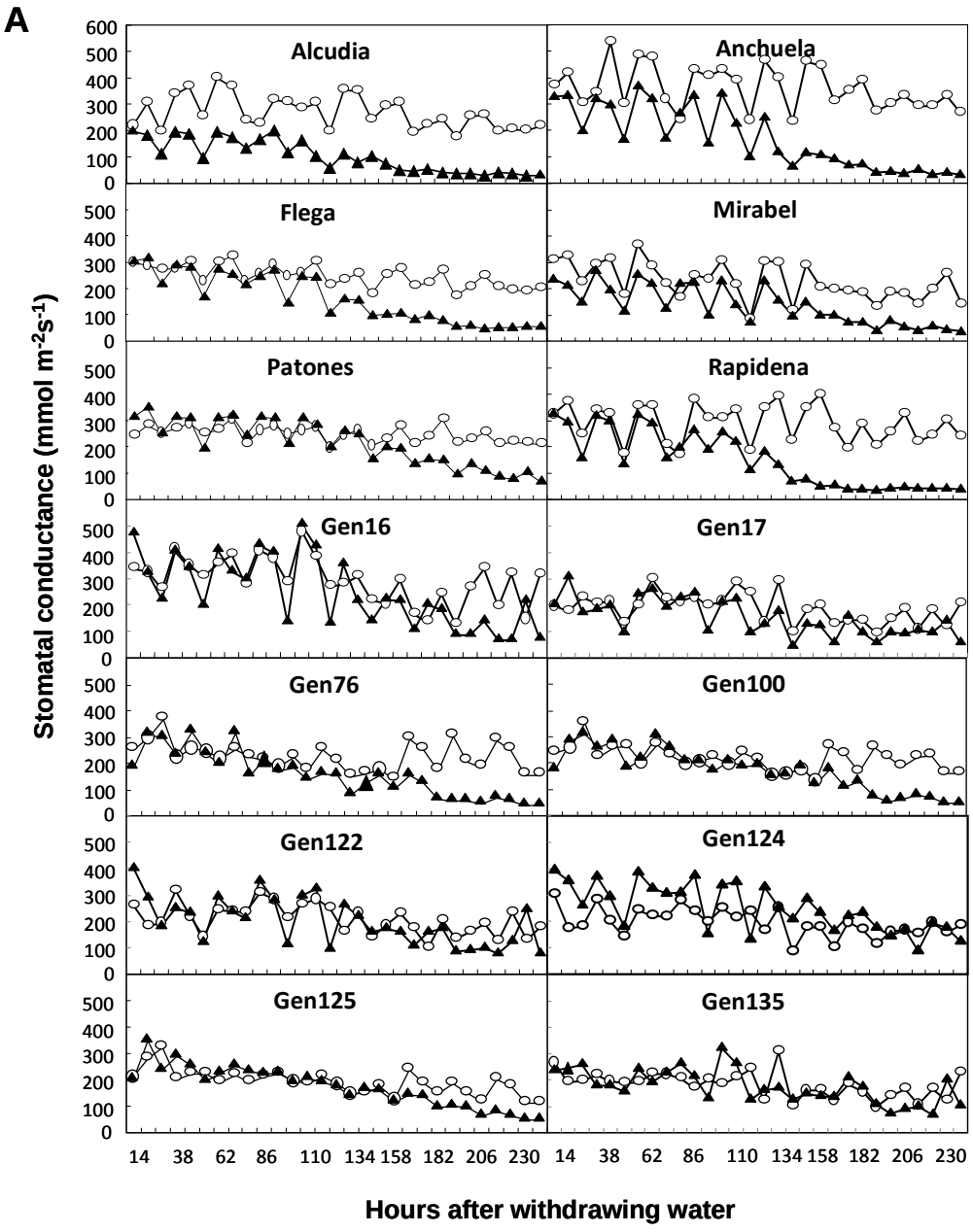

A
Alcudia
Anchuela
Flega
Mirabel
Patones
Rapidena
Gen16
Gen17
Gen76
Gen100
Gen122
Gen124
Gen125
Gen135
Stomatal conductance (mmol m-2s-1)
0
100
200
300
400
500
600
14
38
62
86
110
134
158
182
206
230
Hours after withdrawing water

Figure 3.

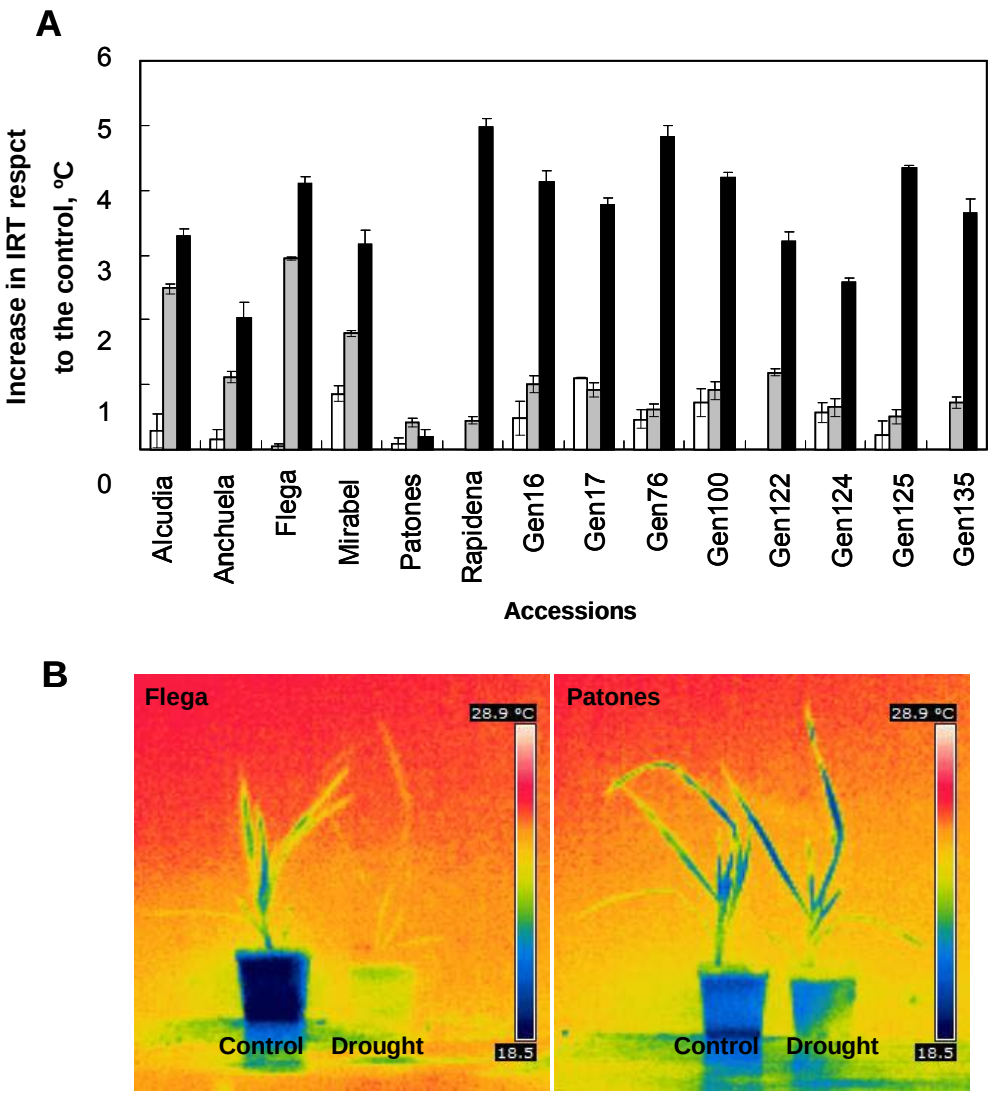

A
Increase in IRT respct to the control, ºC
0
1
2
3
4
5
6
Alcudia
Anchuela
Flega
Mirabel
Patones
Rapidena
Gen16
Gen17
Gen76
Gen100
Gen122
Gen124
Gen125
Gen135
Accessions
B
Flega
28.9 ºC
18.5
Control
Drought
Patones
28.9 ºC
18.5
Control
Drought

Figure 4.

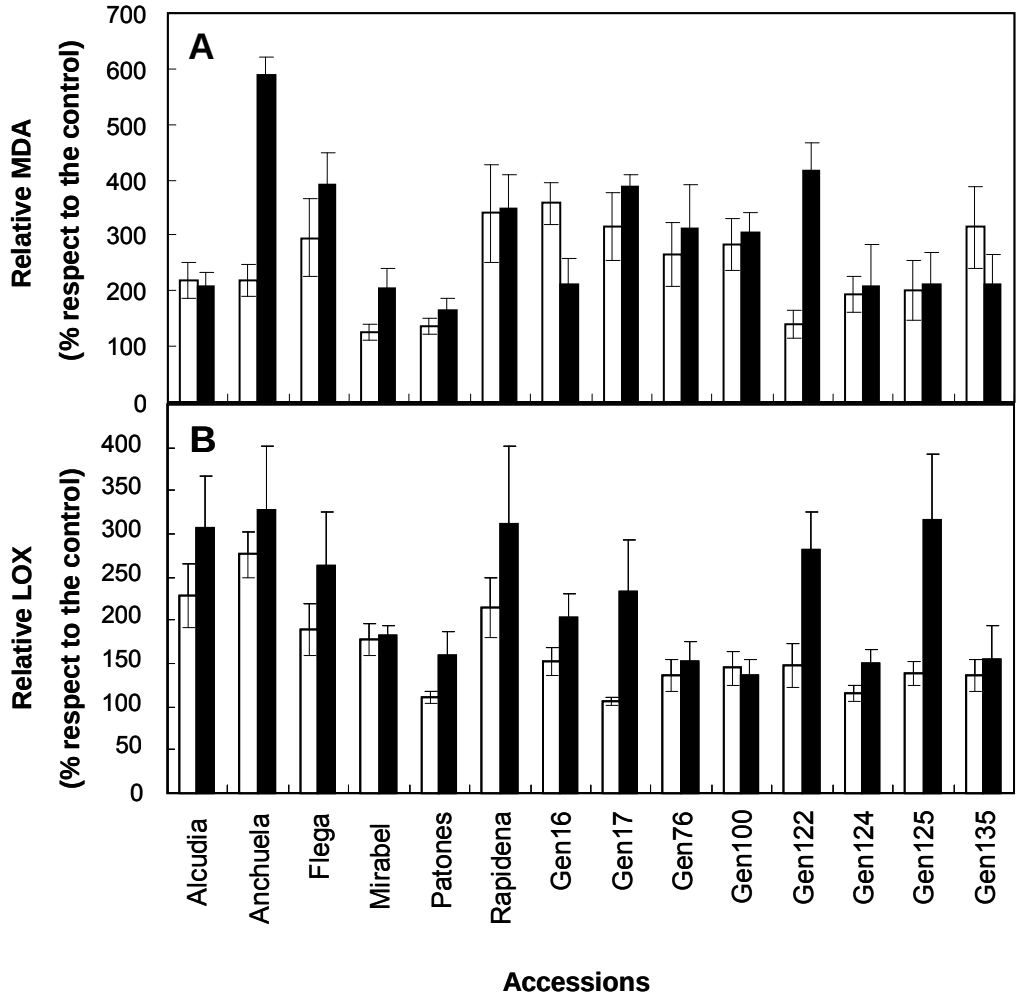

A
B
Relative MDA
(% respect to the control)
Relative LOX
(% respect to the control)
0
100
200
300
400
500
600
700
0
50
100
150
200
250
300
350
400
Alcudia
Anchuela
Flega
Mirabel
Patones
Rapidena
Gen16
Gen17
Gen76
Gen100
Gen122
Gen124
Gen125
Gen135
Accessions

Figure 5.

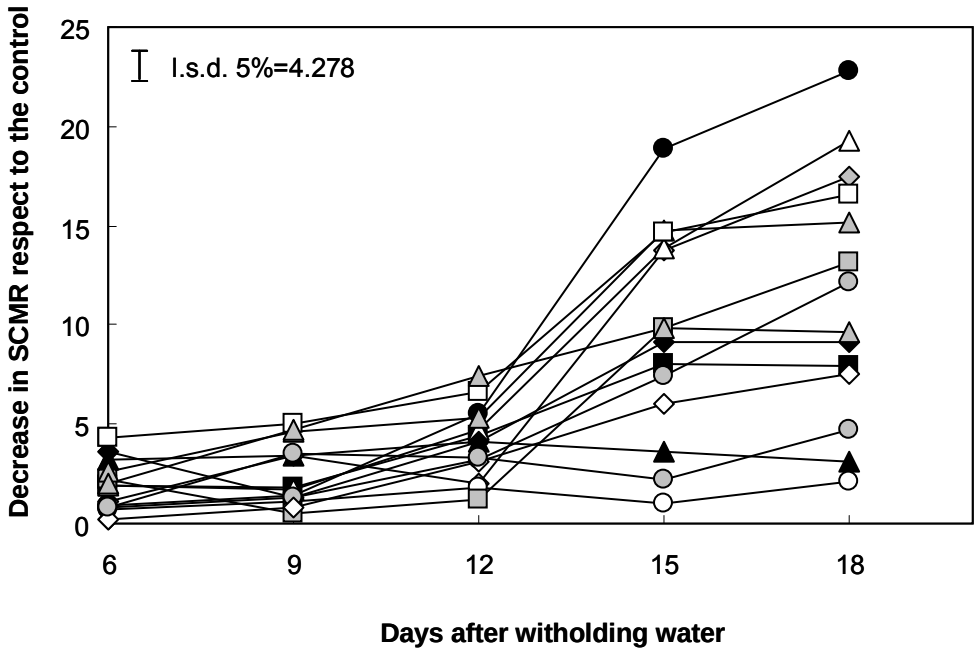

Decrease in SCMR respect to the control
25
20
15
10
5
0
l.s.d. 5%=4.278
6
9
12
15
18
Days after witholding water

Figure 6.

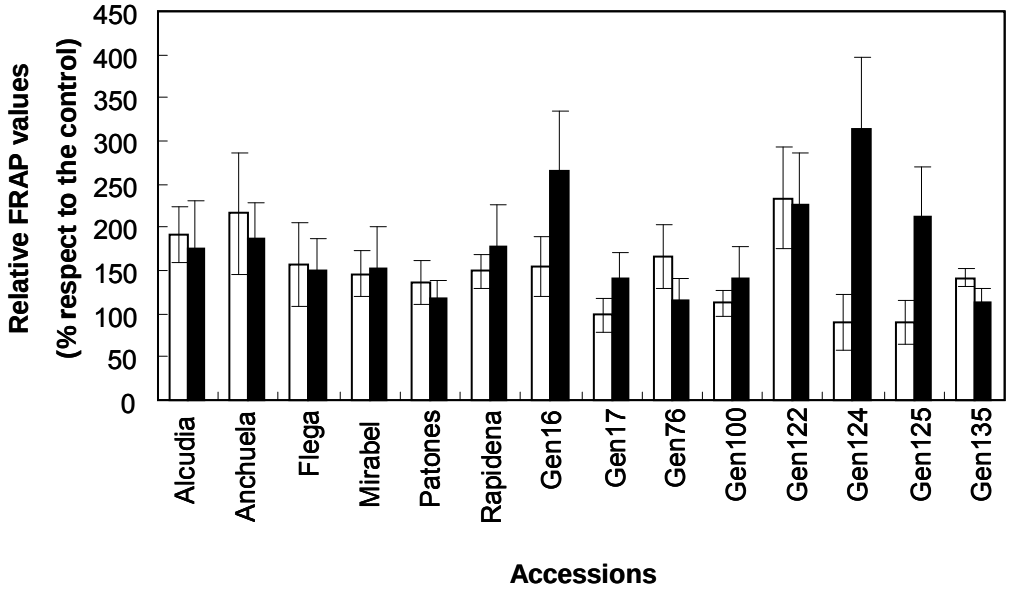

Relative FRAP values
(% respect to the control)
450
400
350
300
250
200
150
100
50
0
Alcudia
Anchuela
Flega
Mirabel
Patones
Rapidena
Gen16
Gen17
Gen76
Gen100
Gen122
Gen124
Gen125
Gen135
Accessions

Figure 7.

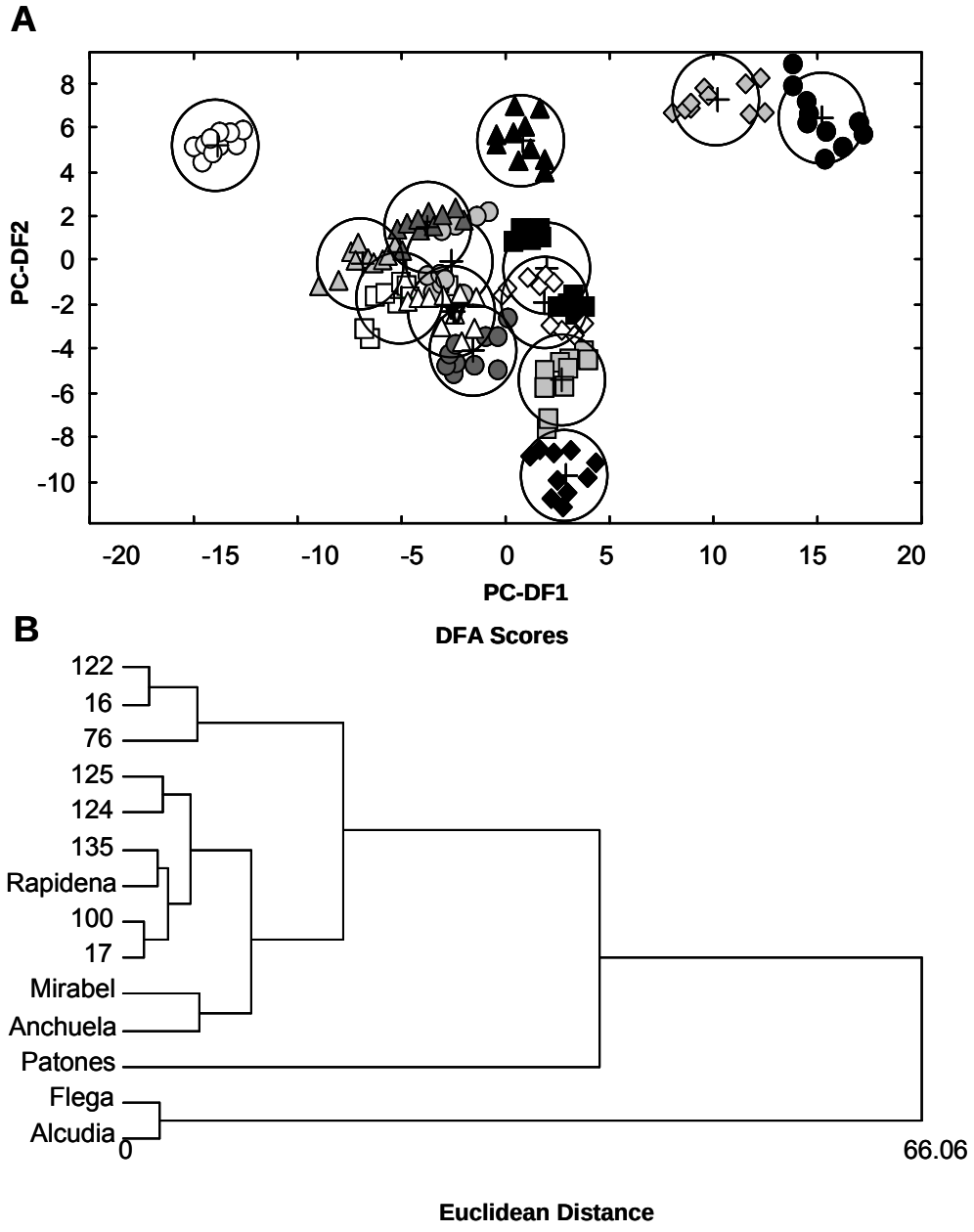

A
PC-DF2
PC-DF1
DFA Scores
B
122
16
76
125
124
135
Rapidena
100
17
Mirabel
Anchuela
Patones
Flega
Alcudia
0
66.06
Euclidean Distance

Figure 8.

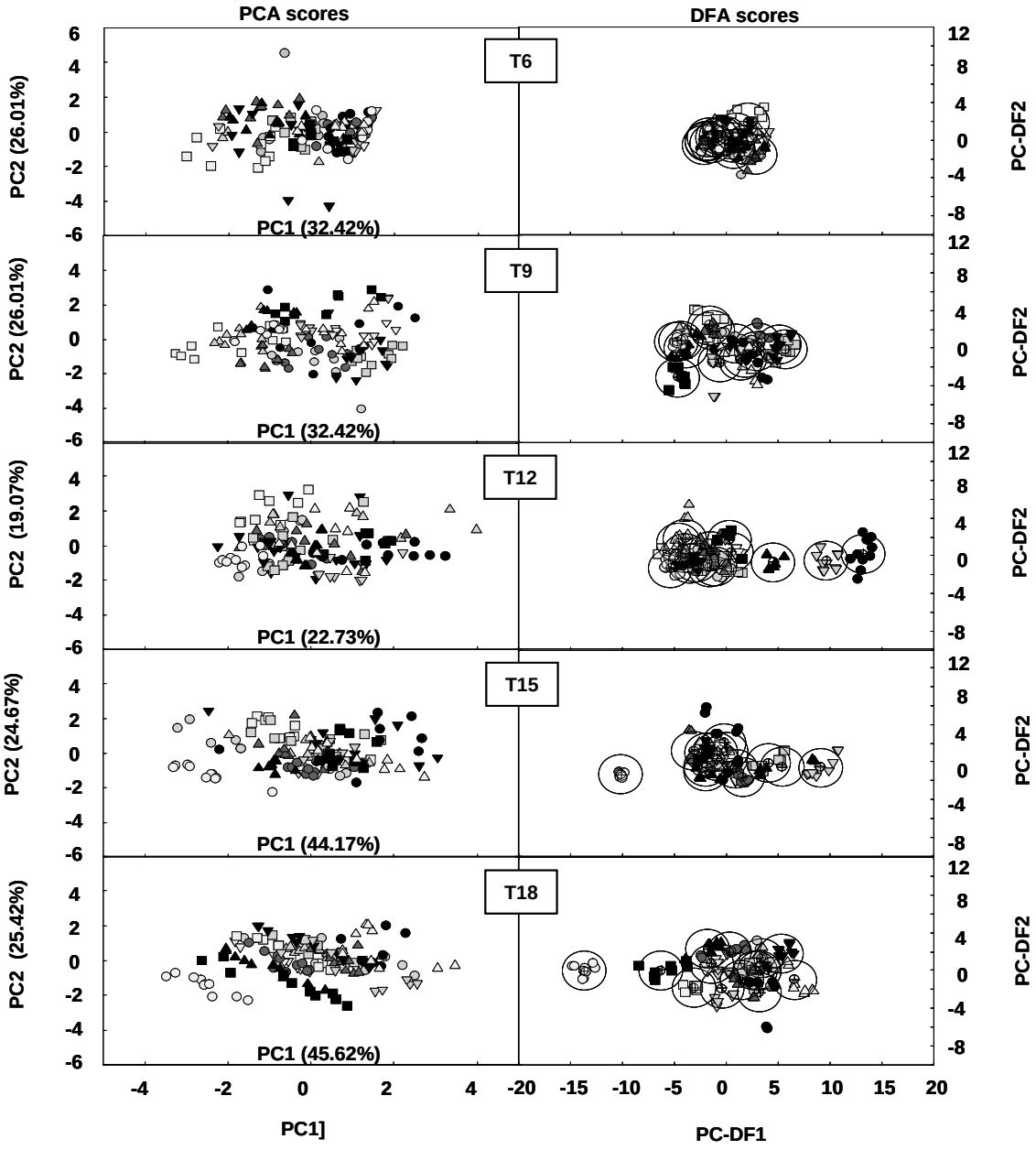

PCA scores
DFA scores
T6
T9
T12
T15
T18
PC2 (26.01%)
PC2 (19.07%)
PC2 (24.67%)
PC2 (25.42%)
PC1 (32.42%)
PC1 (22.73%)
PC1 (44.17%)
PC1 (45.62%)
PC1]
PC-DF2
PC-DF1

Figure 9.

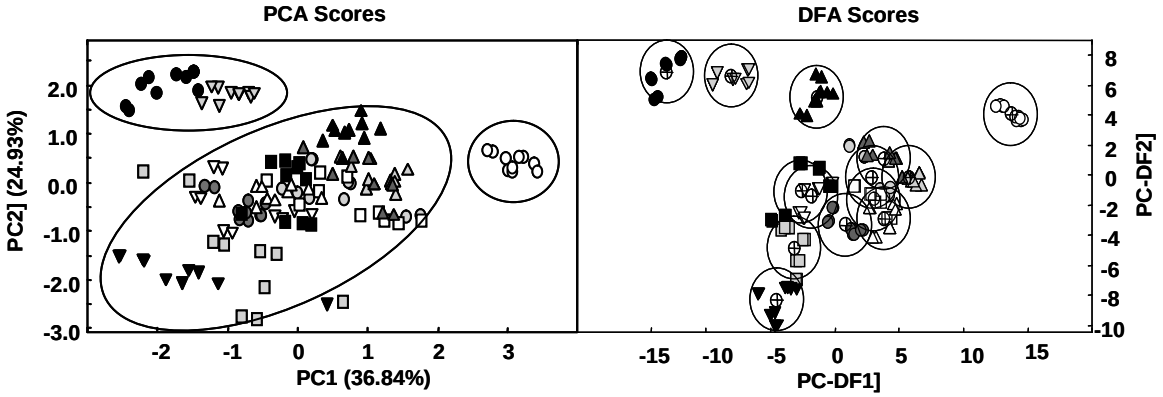

PCA Scores
DFA Scores
PC2] (24.93%)
PC1 (36.84%)
PC-DF2]
PC-DF1]
2.0
1.0
0.0
-1.0
-2.0
-3.0
-2
-1
0
1
2
3
8
6
4
2
0
-2
-4
-6
-8
-10
-15
-10
-5
0
5
10
15

Figure 10.

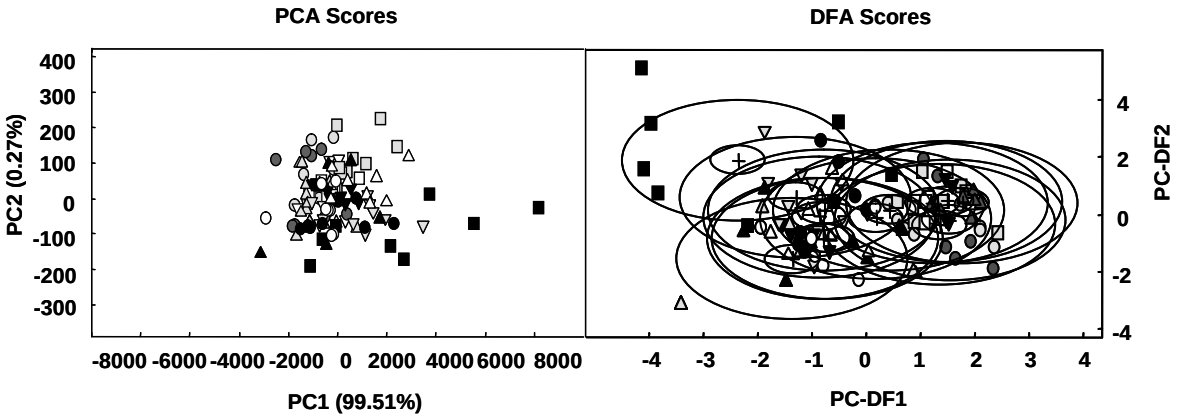
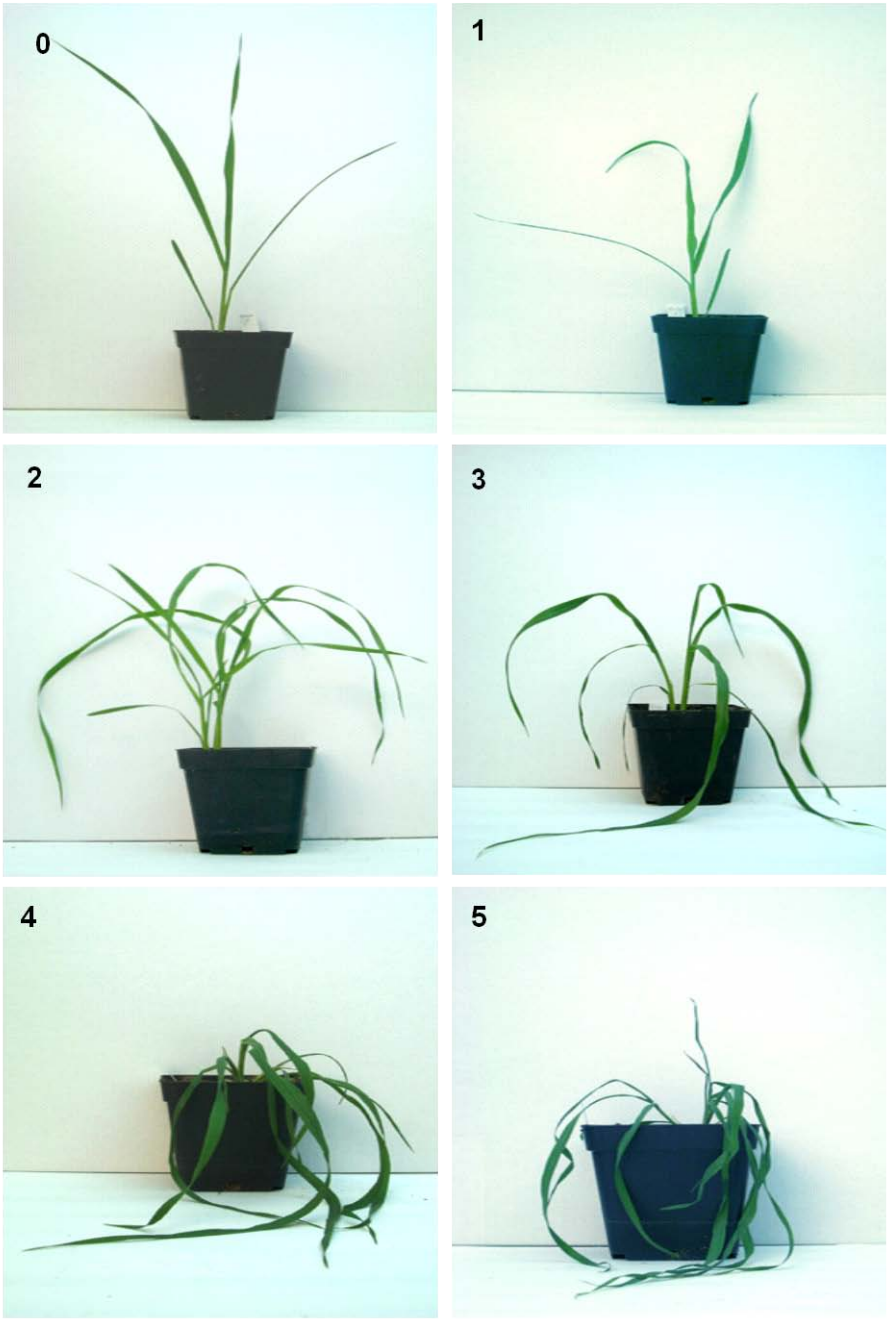

PCA Scores
DFA Scores
PC2 (0.27%)
400
300
200
100
0
-100
-200
-300
-8000 -6000 -4000 -2000 0 2000 4000 6000 8000
PC1 (99.51%)
-4 -3 -2 -1 0 1 2 3 4
PC-DF1
4
2
0
-2
-4
PC-DF2

Figure 11.

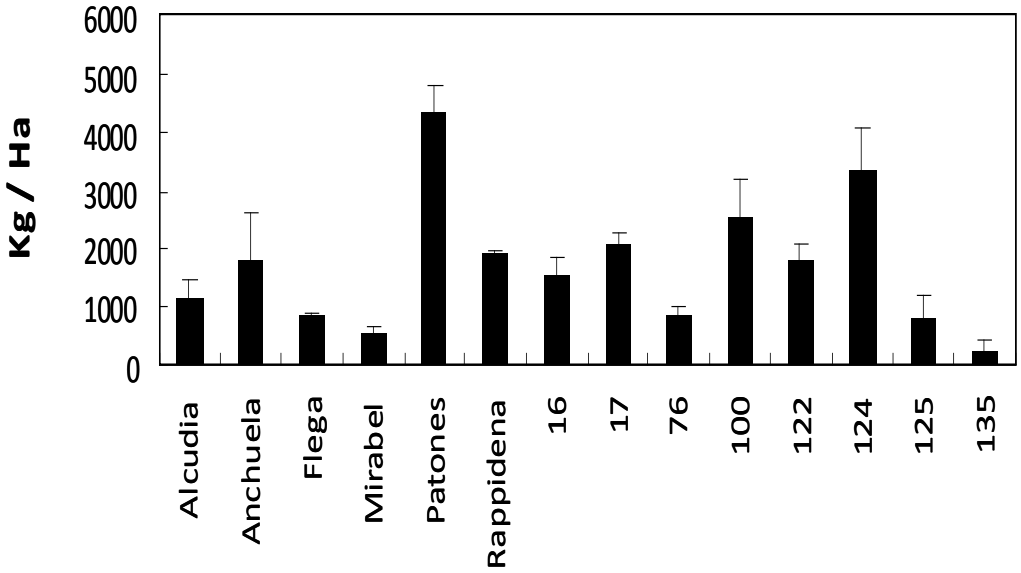
Supplementary Online Resource Legends

Online Resource 1. Visual assessment of drought symptoms during a 19 days time course of drought.
Online Resource 2. Yield (Kg per Ha) of selected genotypes in prone water conditions (242.36mm in the growing season.
Online Resource 3. PC-DFA loading vectors contributing to the derived projections that discriminated between genotypes when assessing **A**. all variables; **B. C.** and **D**. variables taken at 12, 15 and 18 d. a. w. w respectively together with AUDPC and AUCPC taken during the whole time course. The inner and outer circles represent respectively one and two standard deviations from the mean (shown by a cross). Hence, the variables shown represent major sources of variation in the datasets.
Online Resource 4. Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the most susceptible (Flega) and Most resistant (Patones) genotypes based on all variables assessed. Alcudia (▽); Anchuela (■); Flega (●); Mirabel (▲); Patones (○); Rapidena (△; Gen16 (□ Gen17 ( ) Gen76 (▼ Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (⬭).
Online Resource ¡5. Scatterplot of Principal Component Analysis (PCA) and Discriminant Function Analysis (PCA) scores of components 1 and 2 of the moderately resistant genotypes based on all variables assessed. Alcudia (▽); Anchuela (■ Flega (●; Mirabel (▲); Patones (○); Rapidena (△) ; Gen16 (□); Gen17 (▲); Gen76 (▼); Gen100 (○); Gen122 (▽); Gen124 (□); Gen125 (△); Gen135 (⬭).

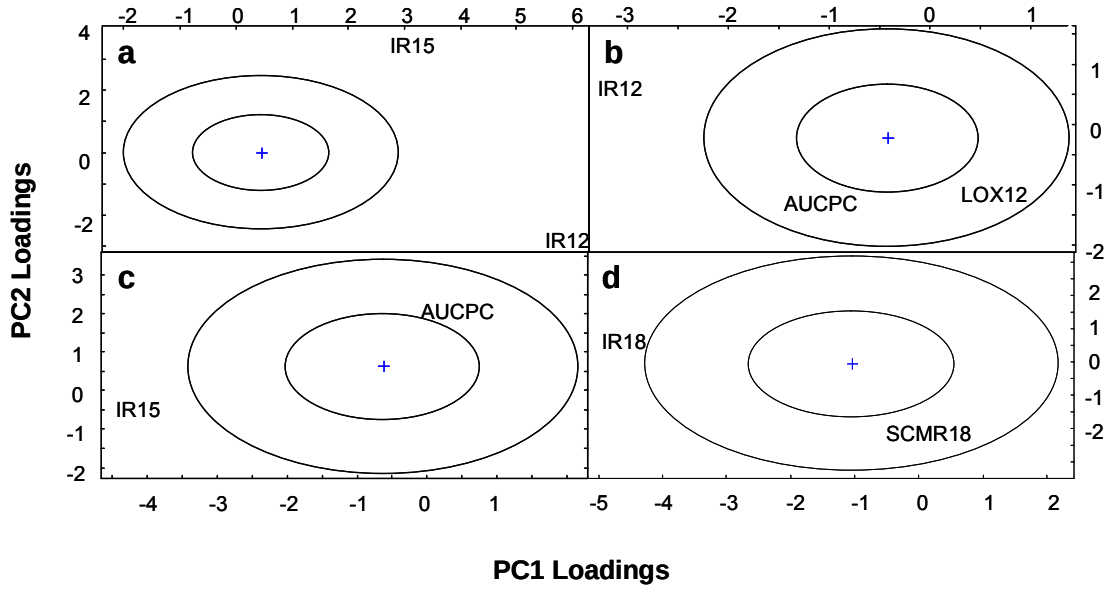
Supplementary Figures



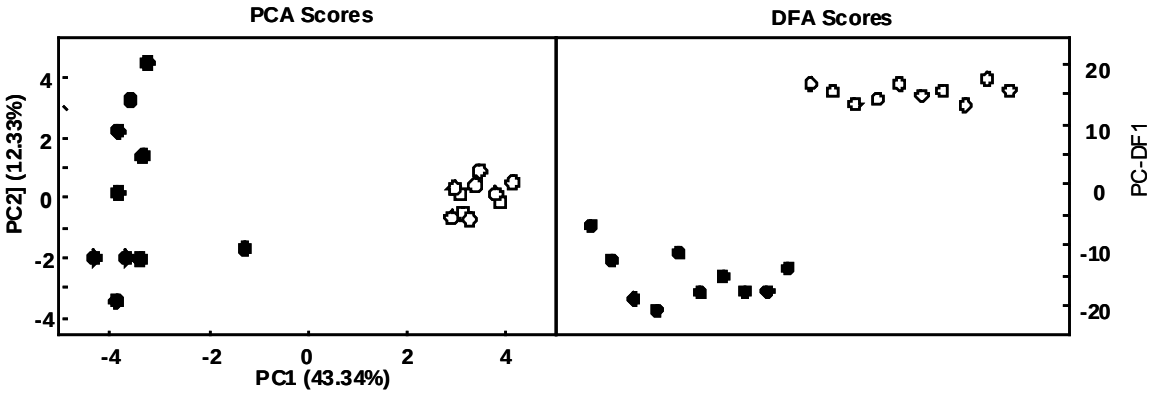
Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.



Supplementary Figure 4.

An integrated mechanism of drought tolerance in oats (*Avena sativa*) is revealed through metabolomic analyses.

Javier Sánchez-Martín[1], Jim Heald[2,], Alison Kingston Smith[2,], Ana Winter[2,], Diego Rubiales[1] , Luis AJ Mur[2], Elena Prats[1].*

[1]CSIC, Institute of Sustainable Agriculture, Apdo. 4084, E-14080 Córdoba, Spain.

[2]Institute of Biological, Environmental and Rural Sciences, University of Aberystwyth, UK

ABSTRACT

Drought is one of the major threats to food security but improving crops for drought tolerance is a complex task for which understanding of drought tolerance mechanisms is essential. We here define metabolomic changes in oat (*Avena sativa* L.) cultivars Flega and Patones, which are, respectively, highly susceptible and tolerant to drought stress. During a time course of increasing water deficit metabolites from leaf samples were profiled using Direct Infusion – Electrospray Mass Spectroscopy (DI-ESI-MS) and analysed using Principal Component Analysis (PCA) and Discriminant Function Anlaysis (DFA). Significant differences in metabolite profiles obtained from genotypes, treatments and sampling times allowed to idenitify 3 main metabolic pathways which differentiates between the responses of Flega and Patones to drought. These were 1) antioxidant pathways; 2) photorespiration and 3) phenylpropanoid metabolism linked to salicylate (SA) biosynthesis. The importance of each pathway was confirmed through targeted assays of key metaboltes. Based on these observations we suggest that the tolerant response of Patones relies on accumulation of; particularly, ascorbate with an induction of photorespiratory pathways to suppress reactive oxgyen species (ROS) to maintain photosynthetic efficiency as was indicated by Fv/Fm measurments. Exogenous application of SA was found to reduce stomatal conductance, so its accumulation in Patones is likely to improve water use efficiency, with any photoinhibitory effect being counteracted by more effiicent antioxidant capacity of this genotype. Thus, our studies suggest that coupled salicylate-and antioxidant defences represent an integrated mechanism of conferring drought tolerance in oats.

INTRODUCTION

Intergovernmental Panel on Climate Change (IPCC) predictions vary from region to region but suggested that many areas would exhibted increased temperatures and severe summer droughts. Other regions would display episodes of intense rainfall in winter and autumn leading to flooding, soil erosion and loss of nutrients (Kumar, 2007). Oats (*Avena sativa*) is attracting increasing interested due to its good adaptation to a wide range of soil types and because it can perform better than other small-grain cereals on marginal soils (Stevens *et al.*, 2004a). However, oats can be sensitive to hot, dry weather and hence, in most Mediterranean and similar climatic regions drought is the main limiting factor for yield (Stevens *et al.*, 2004b). Crop breeders are responding to the challenge of developing new drought tolerant lines. As in most crops, this is achieved,by selection of appropriate progeny. However, selection for complex traits such as stress tolerance, breeding programmes must be based on a sound understanding of innate tolerance mechanisms (Blum, 1999; Dita *et al.*, 2006). Indeed, when breeding drought tolerant crop plants, breeders should not only focus on plant survival but also yield (Turner, 1979). This latter aspect is frequently underplayed so that drought tolerant lines often have considerable yield penalties (Passioura, 2002). Nevertheless, selection on the basis of yield is not appropriate due to its low heritability and a high genotype x environment interaction. This was also suggested from our recent work where we screened high yielding oats varieties for drought tolerance (Sanchez et al, 2012). In conseqeunce, modern breeding strategies attempt to include assessments of physiological, biochemical and molecular characteristics (Araus, 1996; Richards, 1996; Slafer and Araus, 1998) which provide a better understanding of the intricate processes underlying the tolerance response (McWilliam, 1989).

Investigations based on model plants such as Arabidopsis and to a lesser extent in rice have led in a dramatically increased understanding of the molecular bases of drought tolerance (Bartels and Sunkar, 2005). Several hundred genes that respond to drought stress at transcriptional level have been identified (Seki *et al.*, 2002; Yamaguchi-Shinozaki and Shinozaki, 2006). Other targeted molecules include transcription factors such as AREB1/ABF2 (Fujita *et al.*, 2005) and DREB (Dehydration responsive element binding) transcription factor which interacts with specific *cis*-acting DRE/CRT elements present in the promoter region of various abiotic stress-related genes (Dubouzet *et al.*, 2003; Sakuma *et al.*, 2006), protein kinases, such as RPK1, SNF1-related protein kinase 2C, the guard cell-expresed calcium-dependent protein kinases CPK3 and CPK6 (Agrawal *et al.*, 2003; Osakabe *et al.*, 2005; Umezawa *et al.*, 2004; Mori *et al.*, 2006) and dehyhdrin proteins that may aid in stabilising proteins (Rorat, 2006).

Transcriptional and signaling changes act through various biochemical and physiological responses to confer stress tolerance. These responses have been studied in a wide number of species and showi both substantial similarities and dissimilarites in drought tolerance responses; even amongst closely related crops. For instance, physiological responses leading to increased leaf water potential are among the main responses of barley or chickpea to increasing water deficit (Matin *et al.*, 1989; Pannu *et al.*, 1993); however, it is not a defining feature of tolerance in bread wheat (Schonfeld *et al.*, 1988) or faba bean (Ricciardi *et al.*, 2001). Thus, it is necessary to explore the most determinant tolerance responses within for a given species. Biochemical tolerance responses of crops to drought are linked to changes in the metabolic pathways leading to production of 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). These metabolites function as (i) osmolytes to reduce cellular dehydration, (ii) solutes that stabilise enzymes, membranes and other cellular components; and (iii) chelating agents that sequester metals and inorganic ions (Guy *et al.*, 2008). These metabolic changes in response to drought would reflect changes in photosynthesis (Boyer,

1970; Cornic and Fresneau, 2002; Lawlor and Cornic, 2002; Scheibe, 2004) or the activation of futile cycles to prevent over-reduction of photosynthetic electron transport chain components, including those contributing to the photorespiratory cycle and the malate valve (Cornic and Fresneau, 2002; Scheibe, 2004; Asada, 1999) . In addition, chemical signalling also influences growth, the timing of reproduction and stomatal function (Davies *et al.*, 2002; Morison *et al.*, 2008; Sharp, 2002).

Metabolomics represents perhaps the ultimate level of 'omic analysis and within plant sciences is often the target of reverse genetic approaches (Fiehn, 2001, 2002; Hall *et al.*, 2002). Metabolomics will suggest changes in metabolite flux which are linked to only minor changes within either transcriptome or proteome (Urbanczyk-Wochniak *et al.*, 2003). Unlike transcriptomics, metabolomic analysis allows high throughput processing of samples with large numbers of replicates so that statistically satisfying descriptions of many phenomena may be produced (Fiehn, 2001; Goodacre *et al.*, 2004). Given the importance of biochemical changes in the responses of plants to drought, metabolomic approaches would appear to particularly appropriate in elucidating tolerance mechanisms.

In the present work we carried out a metabolite profiling approach by employing Direct Infusion – Electrospray Mass Spectroscopy (ESI-MS) as a tool for the unbiased assessment of metabolic changes in oat leaves in response to drought-stress. We suggest the importance of antioxidant and respiratory pathways in drought tolerance in Patones and demonstrate a novel role for salicylic acid in improving water use efficiency most likely through the promotion of stomatal closure.

MATERIALS AND METHODS

Plant material, growth conditions and sampling

All experiments used the oat cultivars (cvs) Flega and Patones previously characterised as; respectively, susceptible and tolerant to drought stress (Sánchez-Martín et al., 2012). Seeds of cvs. Patones and Flega were kindly provided by the Andalusian Network of Agriculture Experimentation (RAEA) and by the Dr. P. Bebeli (Athens University, Greece) respectively.

As in other cereal drought related studies, experiments were carried out at seedling stage (3 week old plants) (Xiao *et al.*, 2007; Gong *et al.*, 2010; Hao *et al.*, 2009). Seedlings were grown in 0.5 L pots filled with peat:sand (3:1) in a growth chamber with 20 ºC, 65 % relative humidity and under 12 h dark/12 h light with 150 µmol m^{-2} sec^{-1} photon flux density supplied by white fluorescent tubes (OSRAM, Spain). During growth trays carrying the pots were freely watered with a thin layer of water so that approximately 1 cm continuously present in the tray. At day 21, water was withheld from those plants subjected to drought, (Hao et al. 2009; Gong et al. 2010) for a period of 18 days. Control plants were watered as described above during the whole experiment. During the drought time course the relative water content of the soil was monitored daily and by day 18 reached a level of 20% which matched that previously reported for oat work related to drought (Gong et al. 2010).

At set periods, 6, 9, 12, 15 and 18 days, the second leaf of each oat plant were taken from watered and droughted plants, rapidly frozen in liquid nitrogen and lyophilised for metabolomic studies. Each sample consisted on a pool of 4 leaves and six independent replications.Following sampling, the plant was discarded.

Visual assessment of drought symptoms

Drought symptoms assessment were carried out according to Sánchez-Martín et al. (2012). Briefly, drought severity values were assessed daily according to a 0-5 scale where 0 = vigorous

plant, no leaves shows drought symptoms; 1 = one or two leaves show slight drought symptoms (less turgor) but most leaves remain erect; 2 = most leaves show slight levels of drought stress, however one or two leaves still show no drought symptoms; 3 = all leaves show drought symptoms but these are no severe; 4 = all leaves show severe drought symptoms including incipient wilting; 5 = the whole plant is wilted with all leaves starting to dry, rolled and or shrunken. These data were used to calculate the area under the drought progress curve (AUDPC) similarly to the area under the disease progress curve widely used to disease screenings (Jeger and Viljanen-Rollinson, 2001) using the formula:

AUDPC= $\sum_k^{i=1}$ ½ $[(S_i+S_{i+1})(t_{i+1}-t_i)]$

where S_i is the drought severity at assessment date i, t_i is the number of days after the first observation on assessment date i and k is the number of successive observations.

Relative water content

Relative water content (RWC) was measured in five plants per accession according to (Barrs and Weatherley, 1962) . Measurements were carried out in the second leaves. Six hours after the onset of the light period, leaf blade segments were weighed (fresh weight; FW), floated on distilled water at 4 ºC overnight and weighed again (turgid weight; TW). They were then dried at 80 ºC for 48 h. After this, the dry weight (DW) was determined. RWC was then calculated as RWC = (FW - DW) $(TW - DW)^{-1}$ x 100.

Cell membrane stability

CMS was measured in five plants per accession according to (Tripathy *et al.*, 2000). Measurements were carried out in the second leaves. Samples collected were washed three times in deionized water to remove electrolytes adhered on the surface. The samples were then kept in a capped vial (20 mL) containing 10 mL of deionized water and incubated in the dark for 24 h at room temperature. The conductance was measured with a conductivity meter (CMD 510, WPA, UK). After the first measurement the vials were autoclaved for 15 min to kill the leaf tissue and release the electrolytes. After cooling, the second conductivity reading was taken. These two measurements were carried out individually for all the samples from both the control and stress treatments. The control gave a measure of leakage solely due to the cutting and incubation of leaf discs. The conductance of the stress sample was a measure of electrolyte leakage due to water stress and was assumed to be proportional to the degree of injury to the membranes. CMS was calculated as the reciprocal of cell-membrane injury after (Blum and Ebercon, 1981)): CMS% = [(1-(T1/T2)) / (1-(C1/C2))] x 100, where T and C refer to the treated and control samples, respectively; the subscripts 1 and 2 refer to the initial and final conductance readings, respectively.

Stomatal conductance

Leaf water conductance (g_l) was measured in ten plants per cultivar with an AP4 cycling porometer (Delta-T Devices Ltd, Cambridge, UK) according to (Prats *et al.*, 2006) . g_l is the sum of epidermal and stomatal conductance, but as epidermal conductance of oat is low, changes in g_l largely reflect changes in stomatal aperture. The porometer allows rapid measurement that is non-destructive and samples a relatively large area (17.5 x 2.5 mm) of leaf. It was used on the centre of the adaxial surface of leaf laminae. Measurements were carried out in the second leaves three hours after the onset of the light period.

Exogenous application of salicylic acid

To assessed the effects of salicylic acid (SA; 2-hydroxybenzoic acid). a 100 μM SA solution with 1% Tween 20 was sprayed on the entire plants until running off. Control plants were similarly treated with inactive isomer 4-hydroxybenzoic acid (4-hBA). Treatment were applied for the first time at time of withholding water and then daily at the onset of the light period until experiment was completed.

Glyoxylate measurements

Glyoxylate concentration was determined following the method of Rojano et al., (2006) with slight modifications. Second leaves, 100 mg, were ground with a mortar and pestle with liquid nitrogen, and homogenised with 1 mL of pure water. Samples were then extracted by ultrasonication for 10 min at 20 ºC. The extract was centrifuged at 10.000 × *g* for 2min at 4ºC and two 400 μL aliquots were obtained. 45 μL of a freshly prepared 1 % (w/v) solution of phenylhydrazine in 100 mM HCl was added to each aliquot, and those were kept at 60ºC for 10 min. Afterwards, 225 μL of 1 M HCl was added to acidify the extract and finally 90 μL of potassium ferricyanide (1.6 % w/v) was added to one of the aliquot (test aliquot) and 100 μL of pure water to the other aliquot (blank aliquot). Absorbance readings at 532 nm of both aliquots was recorded after 10 minutes in a HT Synergy microplate reader (Biotek, Spain). The glyoxylate content in the samples were calculated as the difference between both readings.

Ascorbate and dehydroascorbate measurements

Ascorbate (AA) and dehydroascorbate (DHA) were determined according to (Foyer *et al.*, 1983) with slight modifications. For the assay, second leaves (100 mg) were ground rapidly to a fine powder and homogenised with cold $HClO_4$ 0.2 M (1:3 w/v). The extract was centrifuged at 14.000 × *g* for 5 min at 4 ºC and supernatant recovered.

For AA determination, 20 μL of the supernatant were added to a mix of 18 0μL of $HClO_4$ 0.2 M and 50 μL of $Na_2H_2P0_4$ 0.1 M. The solution was then neutralised to pH 5.6 with 5M K_2CO_3 (adding around 5 μL per sample) and centrifuged again at 14.000 x r.p.m for 5 min at 4 ºC to remove the precipitate. Ascorbate was measured in the extract by reading the reduction of the absorbance at 265 nm upon the addition of ascorbate oxidase (1.5 units in a 300 μl final volume) in a HT Synergy microplate reader (Biotek, Spain). Total AA concentration in our samples was calculated from the AA standard curve.

For DHA determination, this compound was first reduced to ascorbate following a non-enzymatic reaction and then measured as above (Foyer et al., 1983). Thus, 25 μL of the original extract were mixed with 100 μL of 0.1 M Tricine and 150 μL of 10 mM GSH. Ascorbate was then measured in the extract by reading the reduction of the absorbance at 265 nm upon the addition of ascorbate oxidase (1.5 units in a 300 μl final volume). DHA was calculated as the difference between the ascorbate reading after reduction and the value before reduction.

Glutathione measurements

Reduced (GSH) and oxidised (GSSG) glutahione were measured according to Rahman et al., (2007). Second leaves (250 mg) were ground with a mortar and pestle with liquid nitrogen, homogenized (4:1; w/v) in extraction buffer (0.1 % Triton X-100 and 0.6 % sulfosalicylic acid in 0.1 M KPE buffer, pH 7.5 consisting on 0.1 M KH_2PO_4, 0.1 M K_2HPO_4 and 5 mM EDTA) and centrifuged at 14 000 × *g* for 10 min at 4ºC.

For total GSH determination, 2 0μL of the extract was added to a solution consisting on equal volumes of freshly prepared of DTNB solution in KPE (0.6 mg mL^{-1}) and glutathione reductase (GR) (20 μL of GR [250 units ml^{-1}] in 1.5 mL KPE). After 30 s, for the conversion of GSSG to GSH, 60 μL of β-NADPH in KPE (0.6 mg mL^{-1}) was added. Glutathione content was calculated following the decrease in the absorbance at 412 nm for 2 minutes taking measurements every 20 s. Total GSH concentration in our samples was calculated frorm the GSH standard curve.

For GSSG determination, first GSH in the samples was conjugated with 2-vinylpiridine, then GSSG tranformed to GSH and this was measured as above. To this aim, 2 μl of 0.9 M 2-vinylpiridine in KPE were mixed with 100 μL of extract and incubate 1 h at room temperature. Then, 6 μl of 1.24 M triethanolamine in KPE was added, mixed vigorously and incubated for 10 minutes. The resultant extract was assyed as above for GHS determination taking into account that the measured GSH actually came from the original GSSG in the samples. To determinate the amount of GSH, we used the formula GSH_{TOTAL} = [GSH] + 2 [GSSG].

Chlorophyll fluorescence analysis

Fluorescence quenching analysis were measured using modulated fluorescence on second leaves of dark-adapted plants with a PAM 2100 Fluorometer (PAM-2000; Walz, Effeltrich, Germany). Measurments were made in four different replications according to (Lichtenthaler *et al.*, 2005) and Baker et al., 2008.

The initial fluorescence level (Fo) was determined after dark adaptation (at least 30 min) with a pulsed low red measuring light (ML) (0.1 μmol photons m^{-2} s^{-1}). Then, a 1-s saturating light pulse (aprox. 6.000 μmol photons m^{-2} s^{-1}] of white light was applied to measure the maximal fluorescence (Fm) value. When fluorescence returned again to the F_o level, plants were illuminated by a non-saturating continuous red "actinic light" (AL) (655nm) for 5 minutes to drive photosynthesis and gives F' values. During the AL induced fluorescence kinetics, saturating pulses were applied with 20-s intervals in order to keep track of the fluorescence parameters Fm'(the maximun fluorescence level in the light-adapted state). After several minutes under AL, and when the F value remained low and constant, AL was turned off and a far-red radiation (735 nm) that excited preferentially photosystem (PS) I was switched on during 4 seconds to determine the value of $Fo^{'}$. During the induction kinetic induced by AL the ML is automatically switched to a higher frequency of 100kHz in order to achieve a better signal to noise ratio and time resolution. In ML, while measuring F_0, the frequency of 100 kHz is replaced by 1.6 kHz to avoid any chlorophyll fluorescence induction kinetics. Based on these basic parameters obtained, other parameters with significant physiological relevance i) Maximum Quantum Yield of PSII photochemistry (F_v/F_m) and ii) PSII operating efficiency ($\Delta F/F_m$` also termed F_q'/F_v') were derived.

Dark relaxation measurements

Dark relaxation kinetics were used to determinate the photoinhibitory quench q_I caused by photoinhibiton of PSII units, a major constituent of the non-photochemical quench. Following turning off of the AL during the induction kinetics, a saturation pulse was applied withing the first minute of darkness and then at 5 and 20 minutes. ML remained switched on throughout the dark relaxation measurements of q_N. Then Fm`1, Fm`5 and Fm` 20 were obtained and used to determinate $_NF$values (= Fm-Fm´) and hence q_N ($_NF/F_v$). The photoinhibitory quench, $q_{I,}$ was calculated as $_NF20/F_{v.}$ (Lichtenthaler 2005).

Direct Injection Electrospray Ionisation-Mass Spectrometry (DI-MS) and Tandem MS-MS

The extraction procedure followed that of (Allwood *et al.*, 2006). Analysis was carried out using DI-MS on a Micromass LCT mass spectrometer (Micromass/Waters Ltd., UK) in negative ionisation mode where metabolites are singly ionised by the loss of an H+ , which has been shown to be effective for characterisation of plant extracts (Mattoli et al., 2006). The polar extracts were reconstituted in 0.25 mL 30 % [v/v] methanol: H_2O in 2 mL glass vials with 200 µL inserts (Waters Ltd. UK). Extracts of 10 µL were introduced by DI at a flow rate of 0.5 mL min-1 in 30 % [v/v] methanol: H_2O running solvent, using a Harvard 11 syringe pump (Harvard Ltd., Edenbridge, Kent, UK). The conditions employed were as stated in Johnson et al. (2007). DI-MS data were acquired over the *m/z* range 100-1400 Th and were imported into MATLAB, binned to unit mass and then normalised to percentage total ion as stated in (Johnson *et al.*, 2007) .

MS-MS using collision induced dissociation was performed in the LTQ to provide structural information and aid in metabolite confirmation. Helium was used as a collision gas with an activation mass width of ± 1 atomic mass units. The normalised collision energy was optimised for each metabolite to provide an ion response for the precursor ion of 5-20 % of the base peak in the mass spectrum.

Data analysis

All experiments were performed in completely randomized designs. For ease of understanding, means of raw percentage data are presented in tables and figures. However, for statistical analysis, data recorded as percentages were transformed to arcsine square roots (transformed value = 180/π x arcsin[√(%/100)]) to normalize data and stabilize variances throughout the data range, and subjected to analysis of variance using GenStat 11th Edition, after which residual plots were inspected to confirm data conformed to normality. In addition Shapiro-Wilk test and Bartlett's test were performed to test normality and homogeneity of variances respectively. Significance of differences between means was determined by contrast analysis (Scheffe's).

For multivariate analysis the data were first analyzed using principal components analysis (PCA;(Causton 1987)). PCA and PC-DFA was used as described in Allwood et al., (2006) and followed accepted MSI standards (Goodacre et al., 2007). PCA is an unsupervised method where no *a priori* knowledge of experimental structure is given. Thus, if there is clustering of either 2D or 3D projections of PCA from replicate data, this indicates that the original experimental parameters are the sources of maximal variation. PCA was followed by discriminant function analysis (DFA) which is a supervised projection method (Manly 1994). DFA then discriminated between groups on the basis of the retained PCs and the a priori knowledge of which values were replicates (either biological or machine). DFA was programmed to maximize the Fisher ratio (i.e. the within-class to between-class variance) and the similarity between different classes reflects the optimal number of PCs that are fed into the DFA algorithm. All calculations were performed in Pychem 2.0 (Jarvis et al. 2006).

PC-DFA models were created to identify the key metabolites differentially produced in the susceptible and resistant cultivars. The approach was based on deriving robust models where differences between classes were separated along a particular PC-DF axis. This allowed plotting the contributions of individual metabolites measured to the model ("loading vectors"). Those metabolites that appeared > ± 1 standard deviation (STD) from the mean value loading were recorded as significant metabolites associated with the differences.

Due to the complex experimental structure differing approaches were followed, each generating a list of key metabolites (Supplementary Figure 1). Firstly PCAs and PC-DFA plots were

constructed for genotypes and treatments (4 groups). Separate plots were also constructed for drought treated plants grouped according to the sampling time (14 groups). This identified the key time points and *m/z* to discriminate the susceptible and resistant cultivars. Each *m/z* was tentatively identified accord predicted true mass (*m/z* + 1) and interogration ESI-MS metabolite libraries constructed from the analysis of tomato plants and olive oils (Goodacre *et al.*, 2002) and of the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathways. Further significant predicted metabolites were grouped according to the biochemical pathways. Finally, multivariate analyses of targeted pathways were performed with only the consistuent metabolites to confirm their significance in discriminating drought tolerance responses.

RESULTS

Drought results in greater metabolite changes in oat cv. Flega compared to Patones

The progress of drought symptoms in Flega and Patones following the drought treatment is described elsewhere (Sanchez *et al.,* 2012). Metabolite profiles obtained using DI-ESI-MS were analysed using multivariate approaches determine genotypic, temporal and treatment –associated responses to drought. When profiles for samples from the whole experiment (all timepoints and treatments) were analysed together using non-supervised PCA clustering into biologically relevant groups was not observed (data not shown). As a result, supervised DFA approaches were employed. Crude classification of the results into two genotype and droughted orwatered classes allowed the profiles to be separated into four groups (Fig. 1A). The relative postion of these groups in the DFA biplot suggested that the metabolomic responses to drought compared to watered controls were most pronounced in Flega. Given the susceptibly of this cultivar, was likely to reflect changes associated with a lack of compensatory mechanisms. Further classification according to timepoint and treatment was displayed as DFA plots (Fig. 1B) or as class means using hierachical cluster analysis (Fig. 1C). These indicated greater metabolite changes in Flega which are likely to reflect the effects of drought stress, with the relatively more sublte changes in Patones possibly linked to tolerance mechanisms.

Given the complexity of the data, in order to resolve metabolite changes in each genotype with different treatments separate DFA analyses at each timepoint were undertaken (an example analysis is shown in Supplementary Figure 2. Examination of the loading vectors linked to each *m/z* revealed key differences at each timepoint and the correspondingy likely metabolites were tentatively idendified based on KEGG databases. Table 1 lists metabolites whose contribution to the total variance used to derive the biplot shown in Fig. 1B, was greater than two standard deviations from the mean.. We have recently demonstrated how association of such tentatively identified metabolites with pathways can aid in targeting key biochemical changes since metabolites constituents of a given pathway are likely to be co-regulated (Lloyd *et al.,* 2011). After, examining DFA models discriminating between responses of Flega and Patones to drought for each timepoint as in Supplementary Figure 1 for day 12, significant *m/'z* based on loading vectors (> 1 standard deviation from the mean contribution to total variance) were linked with KEGG pathways. Then the percentage of metabolites ineach pathway which were targeted as being discriinartory as a proportion of the number of metabolites in the whole pathway were calculated (Table 2). Three pathways were noted to have more than 50% of the constituent metabolites having equivalent *m/z* on the list which discriminated between the responses of Patones and Flega to drought (asterisked in Table 2). These observations informed our subsequent analyses.

Patones exhibits increased antioxidant capacity in response to drought stress.

Focusing on the ascorbate-aldarate pathway, data for *m/z* which could be associated with metabolites from this pathway were extracted from the fulll MS dataset. PCA focusing only on this sub-data sets proved sufficent to separate the response of Flega to drought and show distinctiveness from that of Patones (Fig. 2A). Further, time course plots showed distinctive responses in Flega and Patones at different times following withholding of water (Fig. 2B). For the significant metabolites contributing to the observed clusters, mean values for watered samples at a given timepoint were substrated from those of droughted samples at correponding timepoints, analysed using two-way ANOVA and displayed as a pathway associated heat map (Fig. 2C). This indicated that *m/z* linked to ascorbic acid (AA) and dehydroascorbate (DHA) exhibited rapid (6 days after drought) genotype specific responses. These observations suggested that the cellular biochemistry inFlega was more oxidized that Patones during drought stress.

The identity of ascorbate within the samples was confirmed by tandem MS (Supplementary Figure 3 but additionally targeted assays of AA/DHA and GSH/GSSG were assessed in samples from a repeated drought experiment (Fig. 3). In Flega, the imposition of drought resulted in no change in AA nor in a change in the AA/DHA ratio. In contrast in Patones there was an increase in AA with a consequential increase in AA/DHA ratio which was consistent with Patones being more reduced during drought stress. In line with this observation, targeted assays of GSH suggested that the reduced form was predominant in Patones after 15 days of drought whilst the oxidised GSSG form was significantly increased in Flega at the same time. Although not exactly reproducing the results obtained from the main metabolomic experiment (Fig. 2), these targeted assay similarly suggested that Patones possess a higher anti-oxidant capacity.

Photorespiration is elevated during drought stress in Patones

Distruption of electron flow in the chloroplast thylakoid membrane is a major source of ROS during drought (Smirnoff, 1993). Therefore, the photochemical efficiency of chlorophyll was determined by measuring Fv/Fm and operating efficiency of PSII by assessing ΦF/F'm (Fig. 4A). This indicated that Fv/Fm was significantly reduced in Flega at 15 days and ΦF/F'm at 12 days following withdrawl of water but Patones was not significantly affected in either parameter within the time frame of the experiment. Photoinhibition of PSII was also assessed at 18 days following withdrawl of water (Fig. 4B). This showed there was a signifcant increase in photoinhibtion compared to water controls in Flega but not in Patones.

Photorespiratory metabolism (targeted in Table 1) acts to maintain photosynthetic electron flow under drought (Osmond *et al.*, 1997) and could contribute to the prevention of photoinhibition seen in Patones under drought conditions. *m/z* tentatively linked to the metabolites in the photorespiratory pathway were isolated from the full MS spectra for samples for watered and droughted Flega and Patones (Fig. 5A). This subset of data were interrogated by PCA and proved to be sufficient to distinguish between the responses of Flega and Patones to drought (Fig. 5B). Mean values of *m/z* tentatively linked to the photorespiratory pathway were substrated from those of droughted samples at corresponding timepoints. These were analysed using two-way ANOVA and are displayed as heat maps linked to photorespiratory metabolites (Fig. 5C). Comparison of the mean *m/z* values corresponding to particular metabolites for each cultivar suggested that those belonging to the Calvin cycle were the most discrimatory but this complicated interpretation of the data. Therefore, to demonstrate that photorespriatory changes were occuring during drought, the targeted analyses focused on a specific marker metabolite, glyoxylate (Fig. 5D). This assay confirmed the trends observed in the metabolite profiling screen with significant increases in glyoxylate over

water controls in Flega were not seen until 18 days after drought. In contrast, in Patones, significant increases in glyoxylate compared to water controls were observed from 12 days after drought. These data further suggested the earlier deployment of a photorespiratory compensatory mechanism in Patones during drought.

Salicylates accumulation in Patones promotes increased water use efficiency

Another pathways highlighted in Table 2 was the derivation of shikimate and important products such as phenylalanine, which form the first metabolite in the core phenylpropanoid pathway and also the important plant hormone, salicylic acid. *m/z* linked to metabolites forming this pathway (Fig. 6) were extracted and this sub-data set introgated by PCA. PCA based only on these *m/z* was unable to distinguish between the well-watered Patones and Flega but readily discriminated between genotypic responses to drought (Fig. 6A). Examining only the responses to drought in the two genotypes, changes in Flega were poorly defined over time but discrete clustering between the timepoints was noted in Patones (Fig. 6B). Mean values of *m/z* tentatively linked to the shikimate pathway were substrated from those of droughted samples at correponding timepoints. These were analysed using two-way ANOVA and are displayed as heat maps linked to shikimate derivate pathway (Fig. 6C). This could suggest that the there was an early flux of through tryptophan and shikmate to salicylate in Patones.

As salicylic acid (SA)is a well-established stress signal its possible role in confering drought tolerance to oats was investigated. Firstly, the visual effects of exogenous application with salicylic acid (SA) on droughted Patones and Flega were assessed. From the time at which water was withheld all plants were visually assessed for wilting and the extent of wilting scored on a five point scale as described in the Materials and Methods. Application of SA was found to delay drought symptom development in Flega and to a lesser extent Patones (Fig. 7A). This was quantified by calculating the area under the drought progress curve (AUDPC) for each oat cultivar (Sanchez *et al.,* 2012) and revealed significant (P<0.01) reductions in salicilic treated plants during drought stress in both genotypes (Fig. 7A).

One mechanism through which SA could promote drought tolerance could be through stomatal closing. Stomatal conductance was assessed in Patones and Flega with and without exogenous SA application under drought stress or well watered conditions (Fig. 7B). In both watered-controls and droughted plants Flega exhibited signficantly reduced stomatal conductance in response to SA. Interestingly, this was not the case with Patones where exogenous had not additional effect. The area under the progress curve was also calculated for salicilic treated plant under drought stress confirming that SA significantly reduced stomatal conductance, although only in Flega.

The effects of SA on relative water content (RWC) and Cell Membrane Stabilty (CMS) in Flega and Patones was determined (Fig. 7C). In both genotypes SA significantly improved RWC at 14 days after withdrawl of water. However, no impact on CMS was noted in either genotype.

DISCUSSION

Drought is considered the most important stress contributing for yield and economical losses in many regions worldwide for many crops as it is for oats (Farooq et al., 2009). Understanding plant tolerance to drought is therefore of fundamental importance and forms one of the major research topics. Recent work on the model plant Arabidopsis and in a lesser extent on tobacco have dissected

part of the regulatory circuits leading to drought tolerance and have identified stress sensors, transcription factors, promoters, and a large list of genes, proteins and metabolites (Bartels and Sunkar 2005). Despite the apparent success of stress research on these model plants, the findings have been rarely applied to improved crops. Recently, it has been suggested that the imposition of "lethal" drought conditions based on simply withdrawing water; which poorly only reproduces the conditions of environmental imposed drought (Skirycz et al., 2011). This was suggested as a major reason underlying the failure to translate the insights gained from investigations based on model species and/ or controlled environments to the field. This conclusion was directly relevant to our study as we similary established drought conditions by withdrawing water. In mitigation of our approach, it will be noted that we constantly montiored soil water content and that, even at the conclusion of of experimental period, this was never less than 20%. Further, within a Southern Spanish context, prolonged periods where no water is made available to oat crops either via rainfall or irrigation is by no means an unusual situation.

There also remains a question of how far model species can furnish relevant physiological information for oats. Clearly, the best option is to focus studies manly on oats themselves but such studies will be limited due to the relative paucity of genetic information and that mutant/ genetically manipulated lines are often not available that will allow linkage of phenotype with genotype. However, due to the cross-species ubiquity of many metabolites , metabolomic studies are in a particularly strong position to describe large scale physiological events in non-genome verfied organisms and thus it is highly appropriate for using in commercial crops. Further, as the metabolome reflects the summation of transcriptomic, post-transcritpomic and such allosteri controls of biochemical pathways, it may represent the most accurate description of a given biological phenomenon. Indeed, the poor correlation between transcritpomics and protein and enzyme activities with drought has been noted (Pinheiro and Chaves, 2011). Thus, metabolomics should be seen as a highly useful approach for dissecting key routes involved on the interaction of plant and environment (Messerli et al. 2007). This paper describes at metabolomic level drought-induced changes in two oat cultivars, Flega and Patones, previously characterized as susceptible and tolerant to drought respectively (Sánchez-Martín et al., 2012). This current study adopts the mult-iplatform approach to understand metabolite changes as previously describes (Allwood et al., 2006; Lloyd et al., 2011) but also establishes biological validation through targeted assays as a key validatory step.

MS-based metabolomic platform yield information-rich spectra and PC-DFA, showed clear discriminatory clustering between the experimental classes To determine the relative importance of a given metabolite we performed as series of sequential multivariate analyses were employed where comparison was made based on; 1) all classes and time points 2) by treatment, comparing only plants subjected to drought or only control plants and finally; 3) by genotypes, comparing the different timepoints within a genotype.(Supplementary Table 1) This allowed us to select *m/z* values associated with different experimental classes (Table1 and Supplementary Table 1) and determine which pathways were more influenced by the drought treatment (Table 2). Further to confirm the importance of the selected metabolites, we performed again statistical analysis for each of the 14 selected pathways as mentioned above. The "pathway mapping approach" effectively adds another selection criterion, targeting whole pathways rather than individual metabolites. To validate our conclusions, where possible we performed tandem MS-MS using the LTQ Ion trap which involved comparing fragmentation patterns of metabolites with those of standards. However it was not feasible to confirm all the *m/z* within each discriminatory pathway due to a lack of representative standards. For this reason further validation methods for key metabolites were carried out based on physiological (i.e. chlorophyll fluorescence) or targeted biochemical assays. The unbiased metabolomic analyses indicated the importance of three KEGG metabolic pathways with more than 50% of metabolites shown as significant in the discriminatory models. These were the ascorbate-

aldarate pathway, the photorespiration related glyoxylate/dicarboxylate pathway, and the main core of the phenylpropanoid biosynthesis leading to phenylalanine, tryptophan, and SA formation.

The generation of reactive oxygen species (ROS) is an acknowledged feature of drought stress (Cruz de Carvalho, 2008; Kar, 2011; Lee and Park, 2012; Miller *et al.*, 2010). Indeed, over-expression of anti-oxdiant genes has been shown to confer drought tolerance (Prashanth *et al.*, 2008; Wang *et al.*, 2005), It was therefore unsurprising that our data suggested that ascorbate metabolism pathway as one of the most significant discriminating the responses of the tolerant Patones and the susceptible Flega to drought. Indeed, the heat map indicated an induction of the levels of ascorbate and their immediate precursors such as galactono-1,4-lactone and gulono-1,4 lactone in Patones plants subjected to drought respect to control plants (Fig 2). Furthermore direct quantification confirmed an early and significant induction of ascorbate levels in Patones coupled with a high ascorbate/dehydroascorbate rate and showed also, as a longer-time response, high levels of reduced glutathione in Patones compared with a high level of oxidized glutathione in Flega. These high levels of reductans are crucial for the regeneration of ascorbate, which is required for sustained rapid turnover of the water-water-cycle.

The generation of ROS can occur as a consequence of stomatal closure where the maintainance of the light reactions with reduced CO_2 fixation can result in the over-reduction of the photosynthetic components. This may particularly damage ATP synthase which immediate impact on ATP pools (Lawlor and Tezara, 2009). Thus, photoinhibiton is likely to be essential facet of drought stress in the Mediterranean (Pfannschmidt *et al.*, 2009). Indeed, in an analysis of 11 drought adapted Mediterranean species, adaptation to low CO_2 was linked to the maintaince of active Rubisco (Galmes *et al.*, 2011). In C3 plants such as oats, the photorespiratory oxygenation of ribulose-1,5-bisphosphate by rubisco (ribulose-1,5-bisphosphatase carboxylase/oxygenase, Fig 8) is an important component for photoprotection as electrons are transferred from PSII to acceptors within the chloroplast. In addition, excess excitation energy in the PSII antennae may be dealt with by thermal dissipation commonly named non-photochemical quenching (Osmond et al., 1997; Ort and Baker, 2002). However since the proportion of photons thermically dissipated often reaches a maximum well before saturating irradiances are reached, photochemical quenching is a crucial component of photoprotection. Our biochemical./biophysical assays data shows that Flega plants subjected to drought dramatically reduced the relative quantum efficiency of electron transport through PSII, termed PSII operating efficiency. This decrease was not so profound in Patones which avoided reductions of the operating efficiency, maximum quantum yield, and photoinhibition observed in Flega (Fig 4). Indeed in Patones only a small decrease in operating efficiency related to leaf age was observed. The better PSII operating efficiency observed in Patones could be due to 1) the efficiency with which excitation energy is transferred to photochemically active, open, PSII reaction centers, or 2) the ability of PSII to transfer electrons to alternative acceptors, Our metabolomic data suggested predominance of the latter process, as the photorespiratory pathway appeared to be more effectively employed in Patones as suggested by the high levels of *m/z* tentatively identified as representing ribulose-1,5-bisphosphate and phosphoglycolate (Fig 5).). Crucially, direct glyoxylate quantification confirms the early induction of the photorespiration pathway in Patones under drought conditions which not observed in Flega. Thus, our data suggest that when CO_2 concentration was restricted consequence of the stomatal closure photorespiratory mechbanisms in Patones allow the maintainance of electron flux under the drought stress conditions.

An additional component in the Patones drought tolerance pathway was revealed from our targetting of the main core of the phenylpropanoid biosynthesis pathway.. Patones plants subjected to drought exhibited the accumulation of *m/z* which coud represent shikimate and their derivatives, phenylalanine, tryptophan, and SA. Phenylalanine is the precursor of all phenylpropanoid

compounds and indirectly therefore of coumarins, lignins, flavonoids, etc. It is widely acknowledge its role to regulate which influence plant responses to biotic and abiotic stimuli including pathogen attack, low temperatures, drought, UV, low nutrient concentration (Dickson and Paiva, 1995) Recently, it has been shown that activation of a flavin monooxygenase gene YUCCA7 (YUC7), which belongs to the tryptophan-dependent auxin biosynthetic pathway, enhances drought resistance in Arabidopsis (Lee et al., 2012). However, YUC7 would appear to be acting as negative regulatory factor as drought-responsive genes, such as RESPONSIVE TO DESSICATION 29A (RD29A) and COLD-REGULATED 15A (COR15A), are up-regulated in a yuc7 mutant leading to drought tolerance. This was at odds and the increased accumulation of *m/z* which could be linked to the auxin pathway in Patones (data not shown). More telling was our observation that *m/z* tentatively identifed as SA was rapidly accumulating in Patones in response to drought. SA is a well known hormone influencing a wide range of developmental processes and also responses to biotic and abiotic stresses (Vlot et al., 2007 and references therein). Thus SA) has been found to protect plants from heat stress (Dat et al., 1998; Clarke et al., 2004), alleviate cadmium toxicity (Metwally et al., 2003) or harden plants against chilling (Ding et al., 2002). Significantly, SA has recently, been reported to induce stomatal closure along with nitric oxide (NO) production in Arabidopsis (Khokon et al., 2011) and NO mediated stomatal closure have been shown to enhance the adaptative response of wheat to drought stress (García-mata and Lamattina 2001). However no previous reports have directly associated salicylic acid with drought tolerance. Significantly, exogenous application of SA reduced stomatal conductance and increased drought tolerance in Flega. This was not the case with Patones, which could indicate that SA activity in Patones could be near to the maximum threshold at which it exerts its influence.

The accumulation of osmotically active metabolites such as amino acids, glycine betaine, sugars, or sugar alcohols are known to accumulate in the cytoplasm to prevent dehydration (Chen and Murata, 2002). Beyond this, sugars act as substrates and modulators of enzyme activity in carbon-related pathways and via the control of expression of different genes related to carbon, lipid, and nitrogen metabolism. Thus, sugars integrate can integrate cellular responses to internal and environmental alterations. Sugars can also be considered top be signal molecules and impact ABA, auxin and ethylene signalling cascades (Hanson and Smeekens, 2009; Ramel *et al.*, 2009). However, although changes in sugar metabolism were suggested from the metabolomic screen (Table 2; Ko00010; Ko0051;Ko00500) these were not particularly prominent. Targated assays of glucose, fructose and sucrose also suggested no clear, consistent differential difference between Patones and Flega upon the inpostion of drought (Supplementary Figure 3). Thus, at least in the case of Patones, no prominent role for sugar accumulation in establishing drough tolerance.

Nevertheless, based on these our observations it is possible to suggest an integrated mechanism of drought tolerance mechanisms in Patones (Fig.10). Thus, increases in SA results in stomatal closure to maintain high relative water content. The potential photoinhibtory effects of this leading to the generation of ROS are reduced through increase antioxidant capacities as seen by ascorbate metabolism and increasing photorespiration to maintain the photosinthethic electron flux to avoids PSII damage. Contrarywise, in Flega these pathways were not induced or not as earlier as in Patones leading to oxidative damage, reflected early as reduced PSII operating efficiency and photinhibition, that threat the membrane stability and therefore any cellular function (Fig 10). How far SA effects are drought tolerance reflect only stomata centred or has other effects remains to be established.

It will be noted that a recent metanalysis of >450 drought papers has highlighted several of the features that have also been elucidated by our study in oats (Pinheiro and Chaves, 2011). These authors identified photosynthetic perfomance as a major factor in drought tolerance. Thus, reduced stomatal appatures and thus cardon uptake was descibed as influenced photoassimlate partition

which could be related to increassee in shoot/root rations. Photorespiration was again targeted as a mechanism through which electron flow from the light reactions can be dissipated in C3 plants. At the metabolite level drought tolerance could be correlated with changes in sucrose and starch and hormone. However, the authors highlighted the difficulting in obtaining an integrated model for drought tolerance that could be widely applied to many plant species (Pinheiro and Chaves, 2011). In contrast, our metabolomic approaches and have revealed integrated mecchanisms through which Patones which are likely to be conferring drought tolerance. Prominent amongst, these is the maintenance of high relative water content by salicylate-induced regulation of stomatal movements which is the first time described in a cereal crop. This is coupled to photoprotectiive anti-oxidant mechanisms to deal with the increasing oxidative stress promoted by the low CO_2 concentration consequence of the partial stomatal closure (Fig. 10). These observation offer targets – either biochemical or linked genes/markers - which could be exploited in cereal breeding.

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Table 1: Metabolites which contributed most to the differences in drought responses in oat (*Avena sativa*) culitvars Flega and Patones derived in Figure 1.

m/z	Compound	Code	Also significant from individual pathway analysis	Pathway
115	Fumarate	b	Yes	3
129	Agmatine	b	Yes	1
133	3-Dehydro-L-threonate	b	Yes	1
	Malate	b	Yes	2,3
135	L-Threonate	e	Yes	1
136	Anthranilate	d	Yes	3
137	Salicylate	b	Yes	3
147	L-Arabino-1.4-lactone	d	Yes	1
	Oxaloglycolate	d	Yes	2
149	L-Arabinose	a*	Yes	1
	L-Tartrate/meso-tartrate/ dihydroxyfumarate	a*	Yes	2
153	2,3-Dihydroxybenzoate	e	Yes	3
155	Phosphoglycolate	a*	Yes	2
165	L-Xylonate L-Lyxonate	e	Yes	1
167	Phosphoenol piruvate (PEP)	b	Yes	3
169	Glyceraldehyde-3P	a	Yes	3
171	3-Dehydroshikimate	a	Yes	3
173	L-Dehydroascorbate	a	Yes	1
	cis-Aconitate	a	Yes	2
	Shikimate	a	Yes	3
174	Monodehydroascorbate	e	Yes	1
175	L-Ascorbate	a	Yes	1
177	L-Galactono-1.4-lactone	a*	No	1
185	3-phopho-D-glycerate	c	Yes	2
185	Glycerate-3P	c	Yes	3
193	D-Galacturonate	e	No	1
195	L-Galactonate / L-Gulonate	a*	No	1
440	Folate	b*	No	3

a) Significant in discriminating between susceptible and resistant genotypes subjected to drought stress but not in watered controls .

b) Significant discriminating susceptible and resistant genotypes subjected to drought stress but not in watered controls, in two sampling times

c) Significant discriminating susceptible and resistant genotypes subjected to drought stress but not in watered controls, in one sampling time

d) Significant discriminating susceptible and resistant genotypes subjected to drought stress but ALSO watered controls, in at least 4 of the 5 sampling times

e) Significant only in specific time points and/or comparisons

*Significant discriminating susceptible and resistant genotypes subjected to drought stress but not in watered controls in the general model (all timepoints)

Pathways refer to; 1) Ascorbate and alderate. 2) Glyoxylate and dicarboxylate and 3) Biosynthesis of phenylpropanoids.

Table 2: Pathway analysis based on discriminatory metabolites.

KEGG map	Pathway	Metabolites in the pathway	Significant metabolites from models	% significant metabolites
Ko00010	Glycolysis and gluconeogenesis	20	6	30
Ko00051	Fructose and mannose	15	6	40
Ko00052	Galactose	18	7	39
Ko00053	Ascorbate and alderate	24	14	58*
Ko00250	Alanine, aspartate and glutamate	18	4	22
Ko00270	Cysteine and methionine	37	8	24
Ko00330	Arginine and proline	36	15	42*
Ko00380	Tryptophan	52	12	23
Ko00410	beta- Alanine	23	6	26
Ko00480	Glutathione biosynthesis	20	3	15
Ko00500	Starch and sucrose	16	5	31
Ko00630	Simplified Glyoxylate/dicarboxylate	12	6	50*
Map01061	Phenylpropanoids (Main core)	19	10	53*

Supplementary Table 1: Significant compounds highligted by the general model (with all residuals) and related metabolic pathways

m/z	Compound	Pathway	Code	Significant from pathway analyses
109	Catechol	Phenylpropanoids	a	YES
110	Pyrrole-2-carboxylate	Arginine and Proline	a	YES
111	Uracil	beta-alanine and Pyrimidine	a	YES
112	L-1-pyrroline-5-carboxylate	Alanine, Asparate, Glutamine	a	YES
	Thiosulphate	Cysteine	a	YES
	Creatinine / 1-pyrroline-2-carboxylate / L-1-pyrroline-5-carboxylate /	Arginie-Proline	a	YES
115	Fumarate	General metabolism/ Glyoxylate and dicarboxylate	b	YES
116	Betaine /L-aspartate-4-semialdehyde	Glycine-Serine	c	NO
	Indole	Tryptophan	d	NO
	l-aspartate-4-semialdehyde	Cysteine	d	YES
	Guanidino-acetate	Arginine-Proline	d	YES
119	3-methythio-propionate	Cysteine	c	YES
129	Agmatine	Polyamine	b	YES
129	2,5-dioxo-pentanoate	Ascorbate/ Arginine-Proline	b	YES
130	Creatine/ Aminoacetone	Glycine-Serine	c	NO
	2-oxosuccinamate	Alanine, Asparate, Glutamate	c	YES
	n-acetyl-b-alanine	B- Alanine	c	YES
	Glutamate-semialdehyde	Polyamines	c	YES
	creatine / L-glutamate-5-semialdehyde / 2-oxo-5-amino-valerate / norspermidine / n-carbamoyl-putrescine	Arginine-Proline	c	YES
133	3-dehydro-L-threonate	Ascorbate	b	YES
	Malate	Starch/TCA/Phenylpropanoids	b	YES
134	L-homocysteine	Cysteine	d	YES
135	L-Threonate	Ascorbate	e	YES
136	Anthranilate	Tryptophan/ Phenylpropanoids	d	YES
	P-aminobenzoate	Phenylpropanoides		
137	Salyclate	Phenylpropanoides	b	YES
146	Glutamate	Polyamine/ Glutathione /	c	YES
		Photorespiration/ Glyoxylate/dicarboxylate	c	NO
	O-acetyl-L-serine	Cysteine	c	YES
	2-oxo-4-hydroxy-5-aminovalerate	Arginine-Proline	c	YES
147	D-xylonolactone	Pentose phosphate	d	YES
	4-methylthio-2-oxobutanoate	Cysteine	d	YES
	2-dehydro-3-deoxy-arabinoate/ L-	Ascorbate	d	YES
148	Methionine	Polyamines	e	YES

149	L-Arabinose	Pentoses	a *	YES
	L-Ribulose/ L-Xylulose	Pentoses phosphate	a *	NO
	D-xylose	Starch-Glycolysis-		
	L-arabinose	Ascorbate	a *	YES
	4-guanidino-butanoate	Arginine-Proline	a *	NO
150	N-Methylanthranilate	Phenylpropanoids	b *	YES
153	Procatechuate/Dyhydroxybenzoate	Phenylpropanoids	e	YES
154	Histidine	ß-alanine	e	YES
155	2-phosphoglycolate	Photorespiration	a *	YES
	Indole-3-acetonitrile	Tryptophan	a *	NO
156	2-Amino-muconate	Tryptophan	c *	YES
163	L-fucose/ L-Fuculose	Fructose-Mannose /	d	YES
	L-rhamnofuronase/L-rhamnose	Fructose-Mannose	d	YES
165	L-Arabinonate/ L-Lyxonate/ L-Xylonate	Ascorbate	e	YES
	L-Lyxonate/ L-Xylonate	Pentoses Phosphate		
167	Phosphoenolpyruvate	Phenylpropanoids	b	YES
168	3-sulfo-acetate / L-cysteate	Cysteine	b	YES
169	D-glyceraldehyde-3P	Photorespiration / Galactose/ Phenylpropanoids Glyoxylate and dicarboxilate	a	YES
	Glycerone-P	Photorespiration / Pentose		
171	Acetylagmatine	Polyamines	a	YES
	3-Dihydroxyshikimate	Phenylpropanoids	a	YES
173	Arginine	Polyamines/ Arg-Proline	a	YES
	Dehydroascorbate	Glutation / Ascorbate	a	YES
	Indole-3-acetamide/ Indol-3-acetaldoxine	Tryptophan	a	YES
	N-acetyl-ornithine	Arginine-Proline	a	YES
	Shikimate	Phenylpropanoids	a	YES
174	Monodehydroascorbate	Ascorbate	e	YES
	Carboxy-norspermidine	Arginine-Proline	e	YES
	Citruline	Polyamines	e	YES
	N-Acetyl-LAspartate	Alanine-Aspartate-Glutamate	e	YES
175	N-carbomoyls-l-aspartate	Alanine-Aspartate-Glutamate	a	YES
	4,6-Dihydroxy-2,5-dioxohexanoate/ 5-	Pentoses Phosphate	a	YES
	L-ascorbate	Glutathione / Ascorbate	a	YES
	N-hydroxy-tryptamine/Serotonin	Tryptophan	a	YES
	D-glucurono-lactone	Ascorbate	a	NO
177	2-Dehydro-3-deoxy-D-gluconate	Pentose Phospahate	a *	YES
	D-galactono-1,4-lactone/2-Dehydro-3-	Galactose	a *	YES
	Lgulono-1,4-lactone	Ascorbate	a *	NO
181	D-sorbitol	Galactose/ Fructose-mannose	b *	YES
	Galactitol	Galactose	b *	YES
	D-mannitol	Fructose-mannose	b *	YES
185	Ribulose-1,5-bisphosphate	Photorespiration/ Glyoxylate-dicarboxylate	c	YES

	Glycerate 3P/ Glycerate 2P	Starch-Glycolysis-TCA	c	YES
		Phenilpropanoids Glyoxylate/dicarboxylate	c	NO
186	Acetylspermidine	Polyamines	a *	YES
188	N-acetyllisatin	Tryptophan	c	YES
	N-acetyl-glutamate	Arginine-Proline	c	NO
193	Glucuronate/Galacturonate	Starch-Glycolysis-TCA	e	YES
195	D-altronate	Pentoses Phosphate	a *	NO
	D-galactonate	Galactose/ Ascorbate	a *	YES
	L-Gulonate	Pentose phosphate/ ascorbate	a *	NO
196	carbamoyl-P	Arginine-Proline	c	YES
198	O-Phospho-serine	Glycine-Serine met	c	NO
201	Spermine	Polyamines/β-Alanine	e	YES
204	6-hydroxy-kynurenate/ Tryptophane/	Tryptophan	c	NO
206	4-2-aminophenyl-2,4-dioxobutanoate/ 2-Formamino-benzoylacetate	Tryptophan	b	YES
210	5-2-formylethyl-4,6dihydroxypicolinate	Tryptophan	b *	NO
	Creatine-P	Arginine-Proline	b *	YES
211	Isophenoxazine	Tryptophan	e	YES
216	N2-Succinyl Glutamate/Glutamyl-putrescine	Arginine-Proline	e	YES
	B-alanyl-lysine	B-alanine	e	YES
221	Cystathionine	Cysteine	c	YES
		Glycine-Serine	c	NO
226	5-2-carboxy-ethyl-4,6-dihydroxypicolinate	Tryptophan	b	YES
	l-glutamyl-P	Arginine-Proline	b	NO
244	b-alanyl arginine	B-Alanine	c	YES
261	D-Manitol-1P/ D-sorbitol-6P	Fructose-mannose	e	YES
338	D-Fructose-2,6 bisP	Fructose-mannose	e	YES
343	Melibiitol	Galactose	e	YES
440	Folate	Phenylpropanoids	b *	YES
530	CDP-4-keto-3,6deoxy-D-glc	Aminosugars	b *	YES
564	CDP-glucose	Starch-Glycolysis-TCA	d	NO
565	UDP-glucose	Photoresp / Pentoses Phosp /	d	YES
		Starch-Glycolysis-TCA/	d	NO
	UDP-galactose	Galactose	d	YES
579	UDP-D-glucuronate	Pentoses / Starch-Glycolysis-TCA / Ascorbate metab/ Aminosugars	d	YES

Supplementary Table 2: Significant compounds from the analysis of each of the specific pathways arisen from the general analysis but that di not appeared significant in the general analysis**.**

m/z	**Compound**	**Pathway**
101	Cadaverine	Glutathione
103	Malonate	β-Alanine
	Hydroxy piruvate	Photorespiration
117	Succinate	Alanine/ Aspartate/
120	L-Cysteine	Glutathione
131	Oxoloacetate	Starch-Glycolysis-TCA
	Ornithine	Arginine-Proline/ Polyamines/ Glutathione
	N-carbamoyl-β-alanine	B-alanine
	N-carbamoyl sarcosine	Arginine-Proline/ Polyamines
132	Aspartate	Polyamines
140	2-Aminomuconate-	Thryptophan
212	L-4-Aspartyl-Phosphate	Cysteine-methionine
239	Anserine	B-Alanine
247	5- Hydroxyindole-	Thryptophan
258	Galactosamine-6P	Galactose
300	N-Acetyl-D-Galactosamine-	Galactose
586	GDP-4 oxo-6-deoxy-D-mannose	Fructose-mannose

FIGURE LEGENDS

Figure 1. Mulitvarate analysis of metabolite profiles from oat cultivar Patones and Flega following withdrawal of water. A Discriminant Function Analysis (DFA) of metabolite profiles from drought-susceptible Flega (F) and tolerant Patones (P) plants based on 15 Principal Components (PC) explaining > 99.95% of the total variation. w = Control well watered plants; d=drought stressed plants. Circles indicate 95 and 90% confidence intervals with the mean value indicated by a cross. **B.** DFA biplot of PC-DF1 and 2 based on genotype, treatment and time point classes. The numbers refer to 6, 9, 12 ,15 and 18 days after the commencement of the experiment where plants were either regularly watered (w) or water was withdrawn (d). The red ovals group genotypes and treatments and have no mathematical significance. **C.** Hierarchical Cluster Analysis of he selected oat genotypes according to the model represented in Fig 1.b

Figure 2. Analysis of *m/z* tentatively linked to ascorbate pathway in metabolite profiles from oat cultivar Patones and Flega following withdrawal of water. Principal Component Analysis (PCA) of metabolite profiles from drought susceptible Flega (F) and tolerant Patones (P) plants; **A.** comparison of drought (d) and control well watered plants (w); **B.** only of drought treated plants classified according to the time exposed to water stress. The numbers refer to 6, 9, 12 ,15 and 18 days after the commencement of the experiment. **C.** Heat map showing significant changes in *m/z* tentatively linked to the cysteine, methionine and glutathione pathways in Flega and Patones during the experiment.

Figure 3. Glutathione, ascorbate and dehydroascorbate content in suscepible Flega and tolerant Patones plants during a time course of water stress (6, 9, 12 ,15 and 18 days). White bar=control, well watered plants; Black bars= plants exposed to water stress. *, **, *** indicate significant differences at $p<0.05$, 0.01 and 0.001 respectively between control and stressed plants; ns indicates no significant differences.

Figure 4. Chlorophyll fluorescence parameters indicative of photosystem II photochemistry in suscepible Flega and toleran Patones plants. A. Maximum quantum efficiency of PSII photochemistry (Fv/Fm) and Operating efficiency of PSII (ΔF/F'm) in Flega and Patones well watered plants (white circles) and during a time course of water stress (black circles). B. Photoinhibitory quenching of PSII in Flega and Patones well watered plants (white bars) and plants exposed to18 days of water stress (black bars). *, **, *** indicate significant differences at $p<0.05$, 0.01 and 0.001 respectively between control and stressed plants; ns indicates no significant differences.

Figure 5. Photorespiratory pathway analysis in oat cultivars Flega and Patones. Principal Component Analysis (PCA) of metabolite profiles from drought suscepible Flega (F) and tolerant Patones (P) plants comparing **A**. drought (d) and control well watered plants (w) and **B.** comparing drought treated plants. The numbers refer to 6, 9, 12 ,15 and 18 days after the commencement of the experiment). **C**. Heat map showing significant changes in *m/z* tentatively linked to the the glyoxylate/dicarboxylate pahtway in Flega and Patones during a time course of water stress. **D.** Glyoxylate content in suscepible Flega and toleran Patones plants during a time course of water stress (6, 9, 12 ,15 and 18 days). White circles=control, well watered plants; Black circles= plants exposed to water stress. *, **, *** indicate significant differences at $p<0.05$, 0.01 and 0.001 respectively between control and stressed plants; ns indicates no significant differences.

Figure 6. Analysis of m/z linked to salicylate metabolism. Principal Component Analysis of metabolite profiles derived from susceptible Flega (F) and tolerant Patones (P) plants**. A.** comparison of drought

(d) and control well watered plants (w), and **B** of drought treated plants classified according to the time exposed to water stress (6, 9, 12 ,15 and 18 days). **C.** Heat map showing significant changes in m/z tentatively linked to the salicylate metabolism in Flega and Patones during the experiment.

Figure 7. Effect of salicylate on drought symptoms of Flega and Patones plants exposed to water stress. **A**. White circles=control plants; black circles=salicylate treated plants. **B.** Stomatal conductance of Flega and Patones, well watered and exposed to water stress, plants. White circles=control plants; black circles=salicylate treated plants. **C.** Relative water content and cell membrane stability of Flega and Patones plants exposed to water stress. White bars=control plants; black bars=salicylate treated plants

Figure 8: An integrated model of drought tolerance in oat culitvar Patones as compared response in the susceptible cultivar Flega. The schematic brings together the physiological and metabolomic observations described in this paper. Changes in the relative levels of ascorbate/ dehydroxyascrobate indicates increases in oxidative stress at 6 days after withdrawing water in Flega but not Patones where ascorbate remains unchanged during drought (Figure 3). In Patones, the synthesis of salicylic acid (Fig.) closes the stomata to reduce water loss through transpiration (Fig.). The resulting photooxidative stress is reduced through the employment of photorespiratory dissipation of the excess excitation energy (EEE; Fig), thus membrane damage is minimized. By contrast, stomatal closure is relatively delayed in Flega and photorespiration dissipation of EEE is less efficient that Flega. The resulting oxidative stress compromises photosynthetic electron flow and reduces photosynthetic efficiency as well as leading to considerable membrane damage.

FIGURES

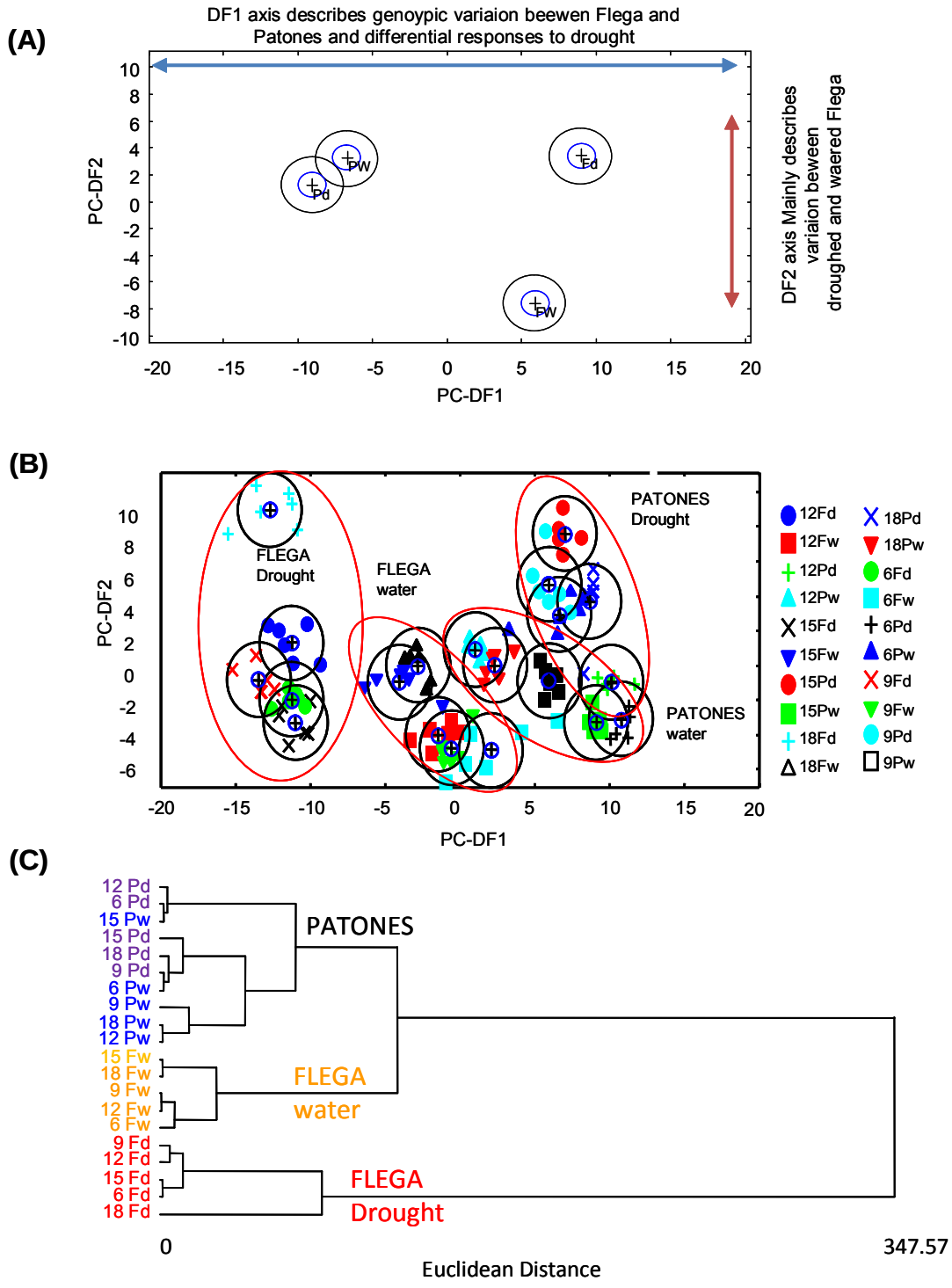


Figure 1.

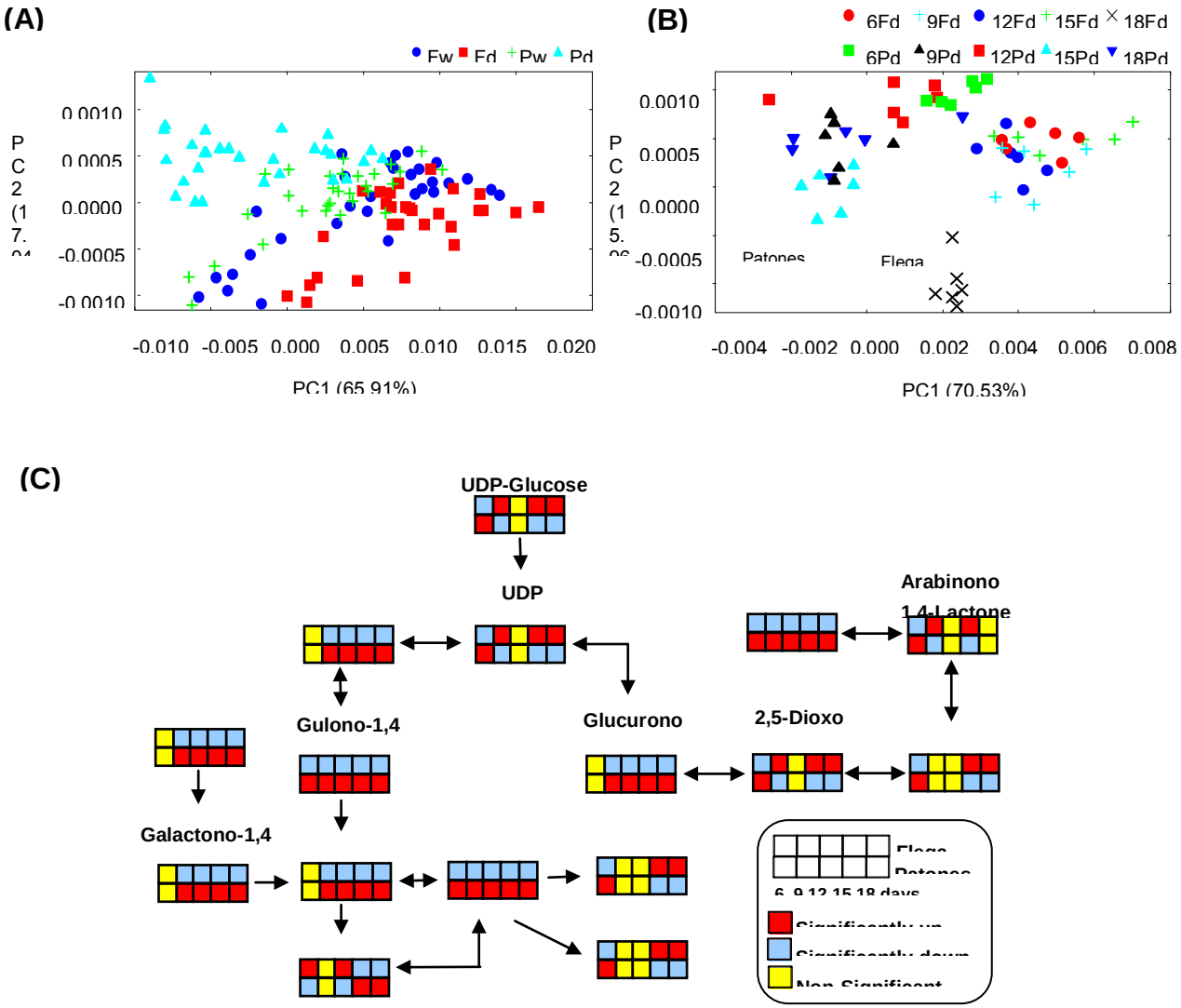

(A)
Fw Fd Pw Pd
PC2 (17.04)
0.0010
0.0005
0.0000
-0.0005
-0.0010
-0.010 -0.005 0.000 0.005 0.010 0.015 0.020
PC1 (65.91%)
(B)
6Fd 9Fd 12Fd 15Fd 18Fd
6Pd 9Pd 12Pd 15Pd 18Pd
PC2 (15.06)
0.0010
0.0005
0.0000
-0.0005
-0.0010
Patones
Flega
-0.004 -0.002 0.000 0.002 0.004 0.006 0.008
PC1 (70.53%)
(C)
UDP-Glucose
UDP
Arabinono
1,4-Lactone
Gulono-1,4
Glucurono
2,5-Dioxo
Galactono-1,4
Flega
Patones
6 9 12 15 18 days
Significantly up
Significantly down
Non Significant

Figure 2.

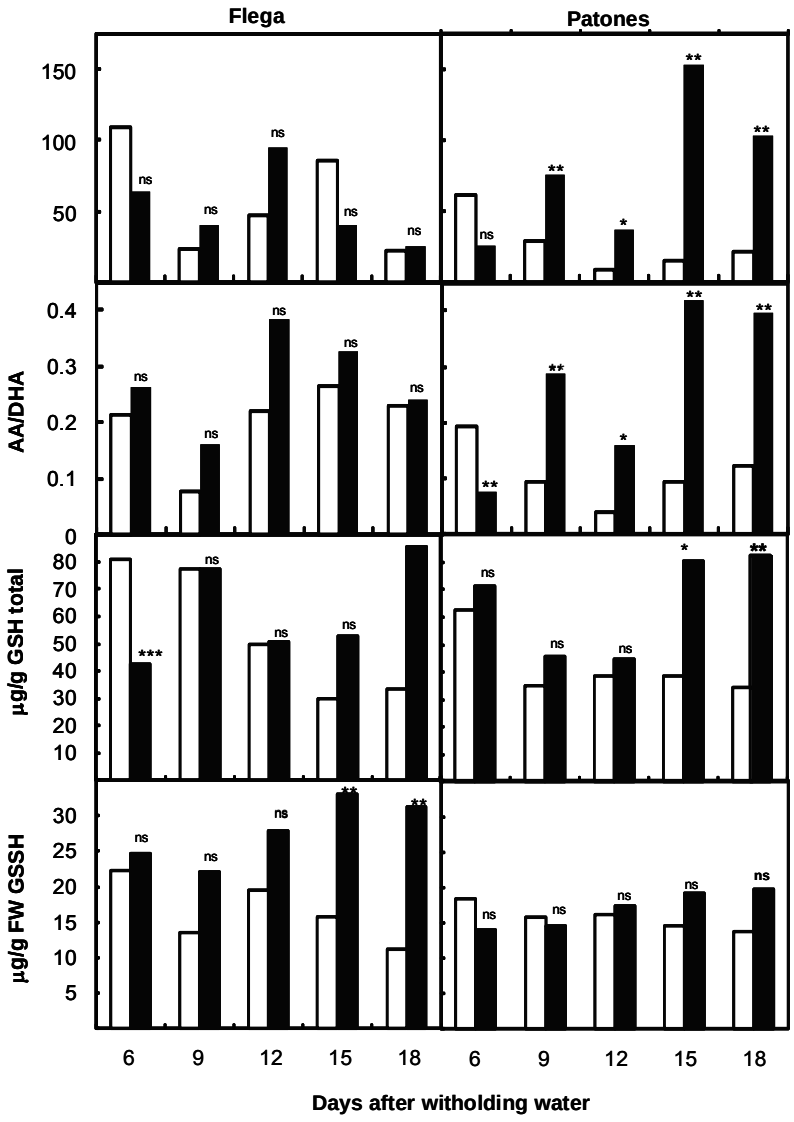

Flega
Patones
AA/DHA
µg/g GSH total
µg/g FW GSSH
Days after witholding water
6
9
12
15
18

Figure 3.

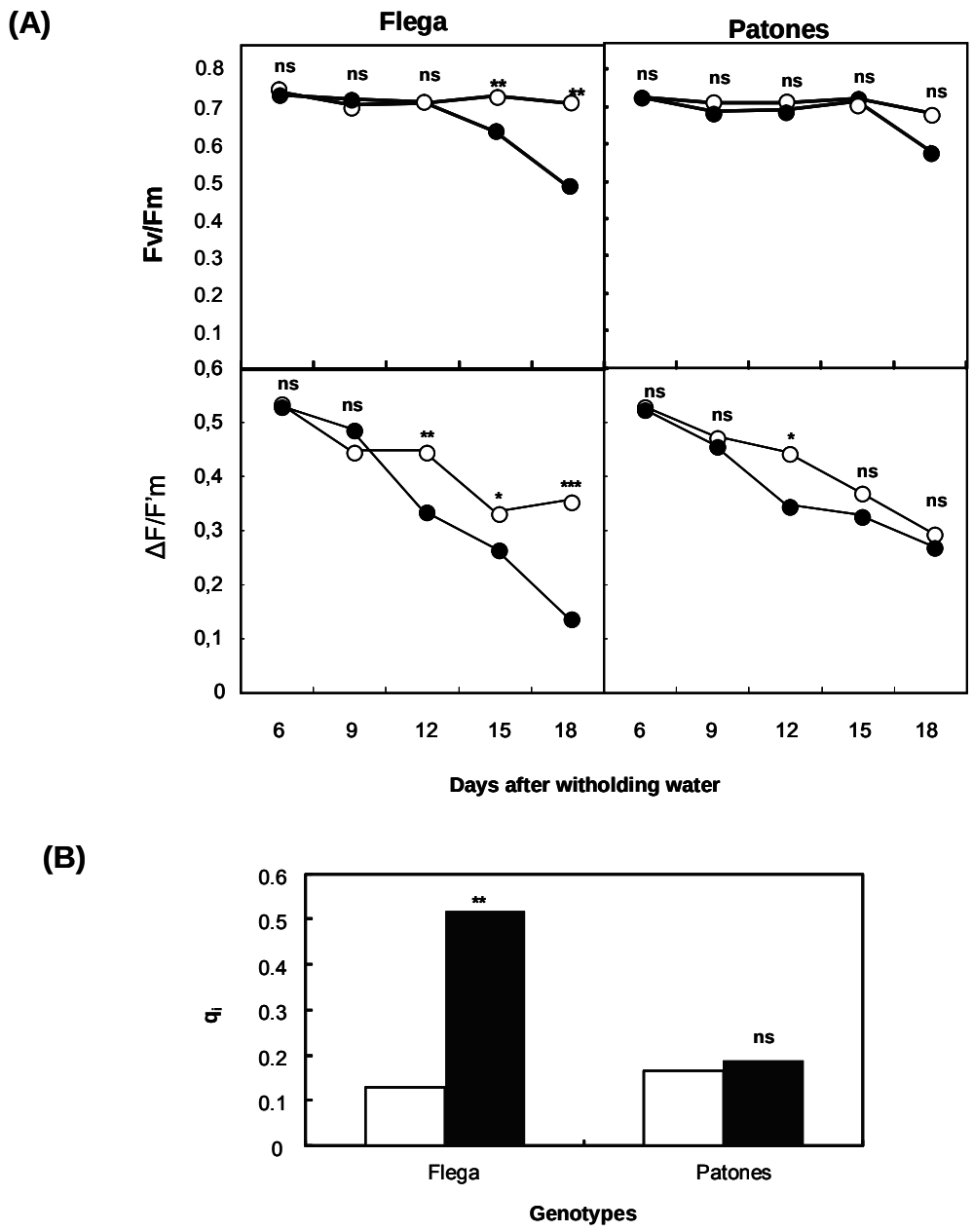

(A)
Flega
Patones
Fv/Fm
ΔF/F'm
Days after witholding water
(B)
Genotypes
Flega
Patones

Figure 4.

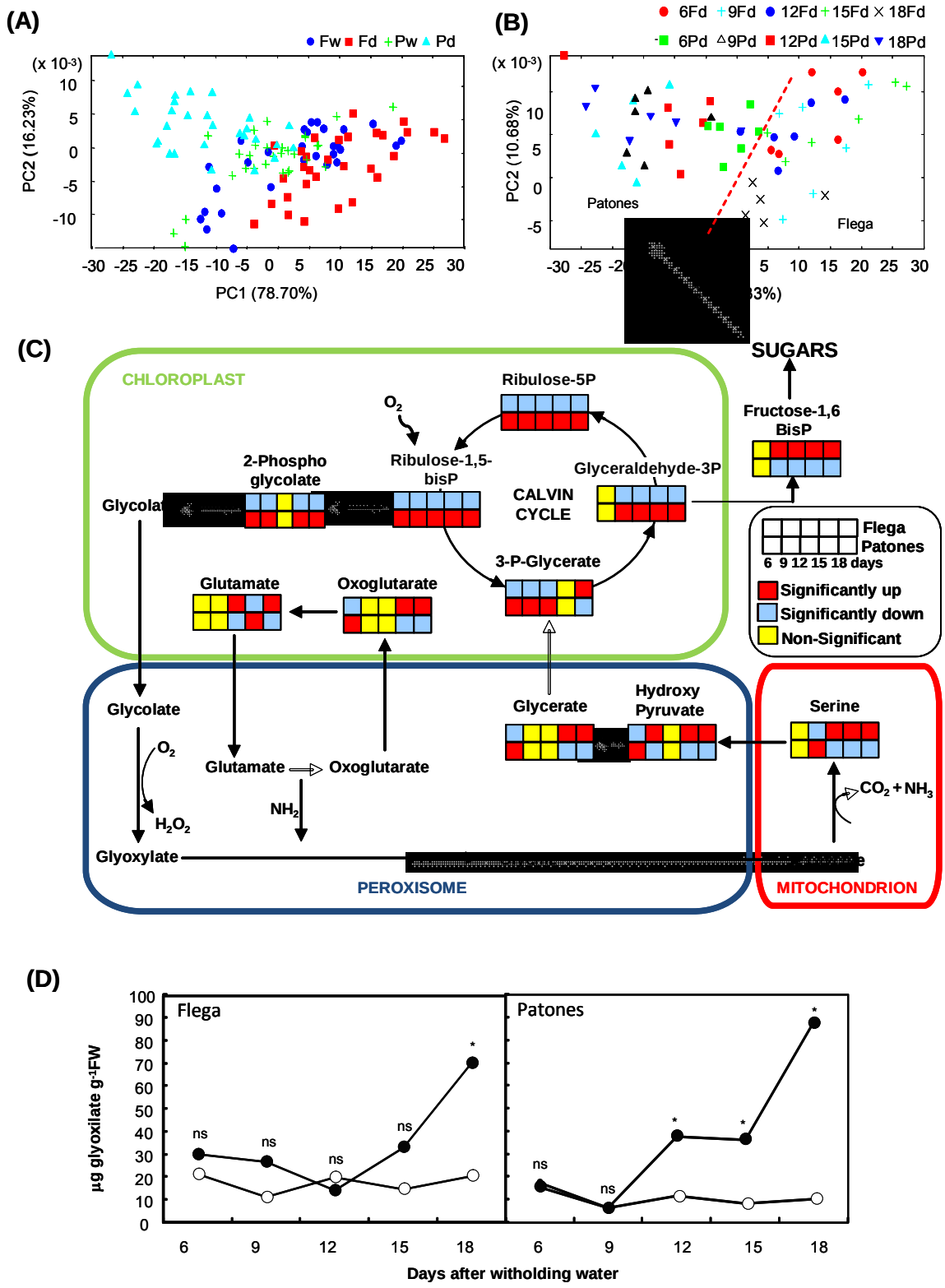

(A)
Fw Fd Pw Pd
(x 10-3)
PC2 (16.23%)
PC1 (78.70%)
(B)
6Fd 9Fd 12Fd 15Fd 18Fd
6Pd 9Pd 12Pd 15Pd 18Pd
(x 10-3)
PC2 (10.68%)
Patones
Flega
(C)
CHLOROPLAST
SUGARS
Ribulose-5P
O2
Fructose-1,6 BisP
2-Phospho glycolate
Ribulose-1,5-bisP
Glyceraldehyde-3P
CALVIN CYCLE
Glycolat
3-P-Glycerate
Glutamate
Oxoglutarate
Flega
Patones
6 9 12 15 18 days
Significantly up
Significantly down
Non-Significant
Glycolate
Glycerate
Hydroxy Pyruvate
Serine
O2
Glutamate
Oxoglutarate
CO2 + NH3
NH2
H2O2
Glyoxylate
PEROXISOME
MITOCHONDRION
(D)
Flega
Patones
µg glyoxilate g-1FW
ns
Days after witholding water

Figure 5.

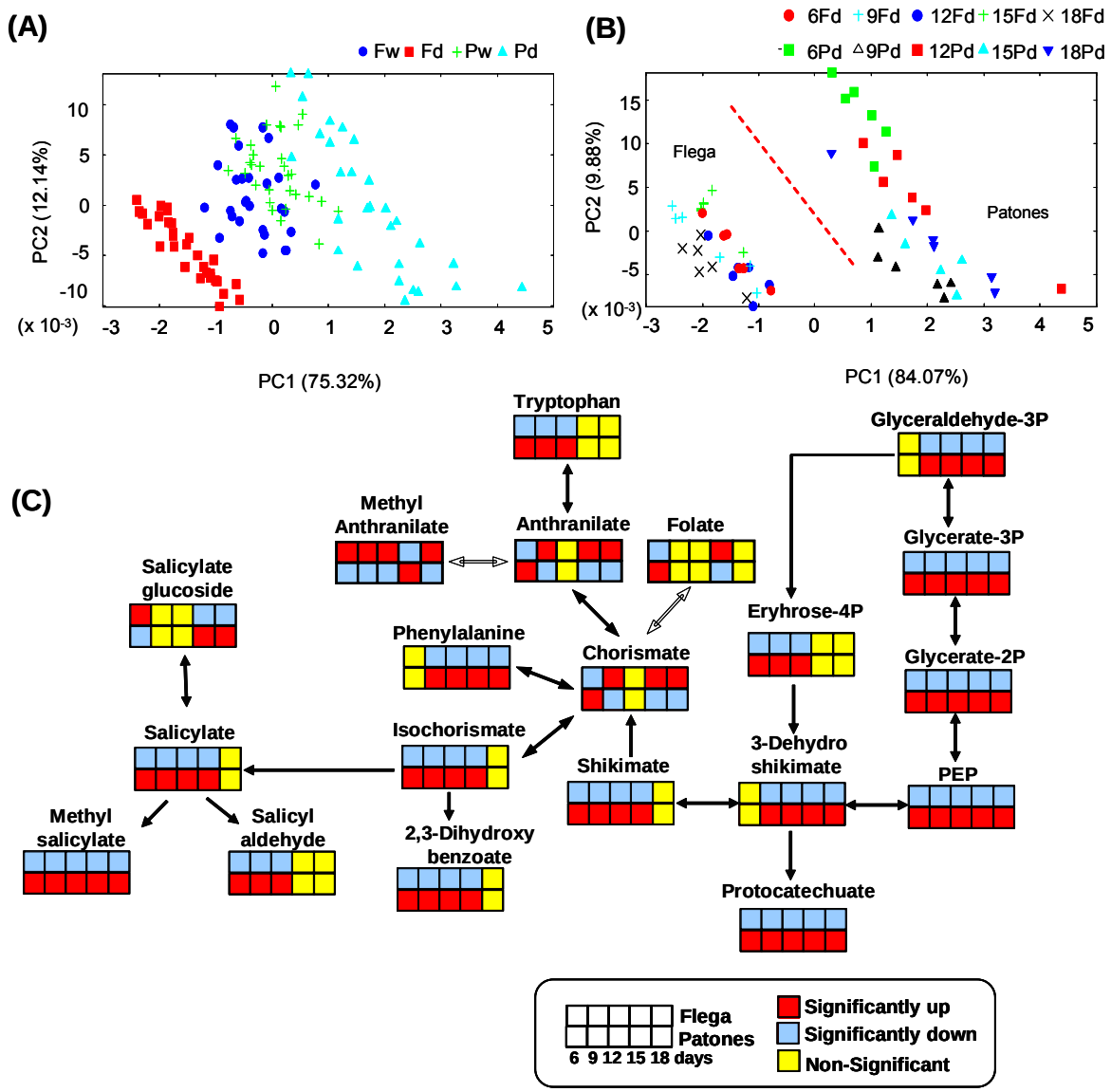

(A)
Fw Fd Pw Pd
PC2 (12.14%)
(x 10-3)
PC1 (75.32%)
(B)
6Fd 9Fd 12Fd 15Fd 18Fd
6Pd 9Pd 12Pd 15Pd 18Pd
Flega
Patones
PC2 (9.88%)
(x 10-3)
PC1 (84.07%)
(C)
Tryptophan
Glyceraldehyde-3P
Methyl Anthranilate
Anthranilate
Folate
Glycerate-3P
Salicylate glucoside
Phenylalanine
Chorismate
Eryhrose-4P
Glycerate-2P
Salicylate
Isochorismate
Shikimate
3-Dehydro shikimate
PEP
Methyl salicylate
Salicyl aldehyde
2,3-Dihydroxy benzoate
Protocatechuate
Flega
Patones
6 9 12 15 18 days
Significantly up
Significantly down
Non-Significant

Figure 6.

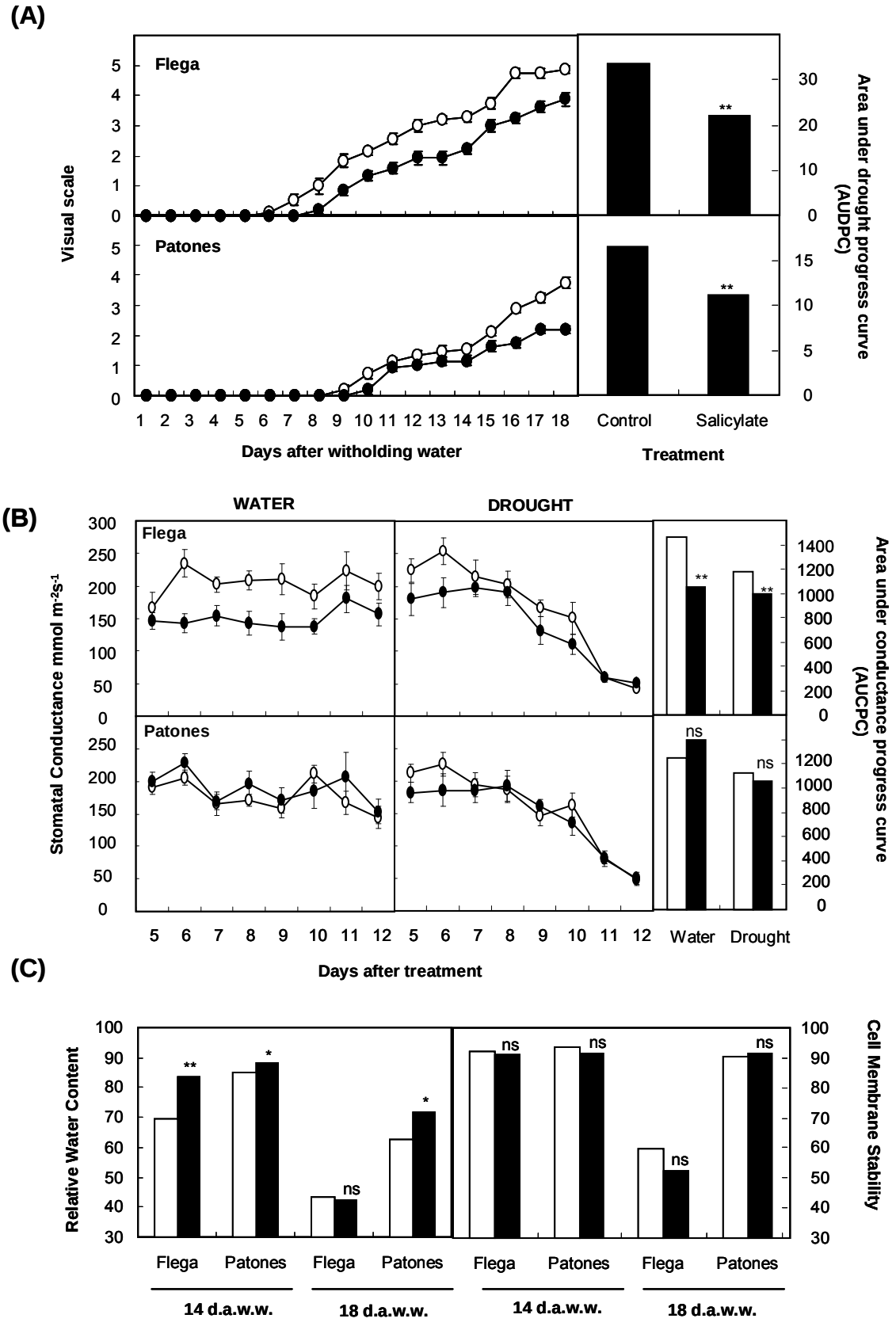

(A)
Flega
Patones
Visual scale
Days after witholding water
Control
Salicylate
Treatment
Area under drought progress curve (AUDPC)
(B)
WATER
DROUGHT
Flega
Patones
Stomatal Conductance mmol m-2s-1
Days after treatment
Water
Drought
Area under conductance progress curve (AUCPC)
(C)
Relative Water Content
Cell Membrane Stability
Flega
Patones
14 d.a.w.w.
18 d.a.w.w.

Figure 7.

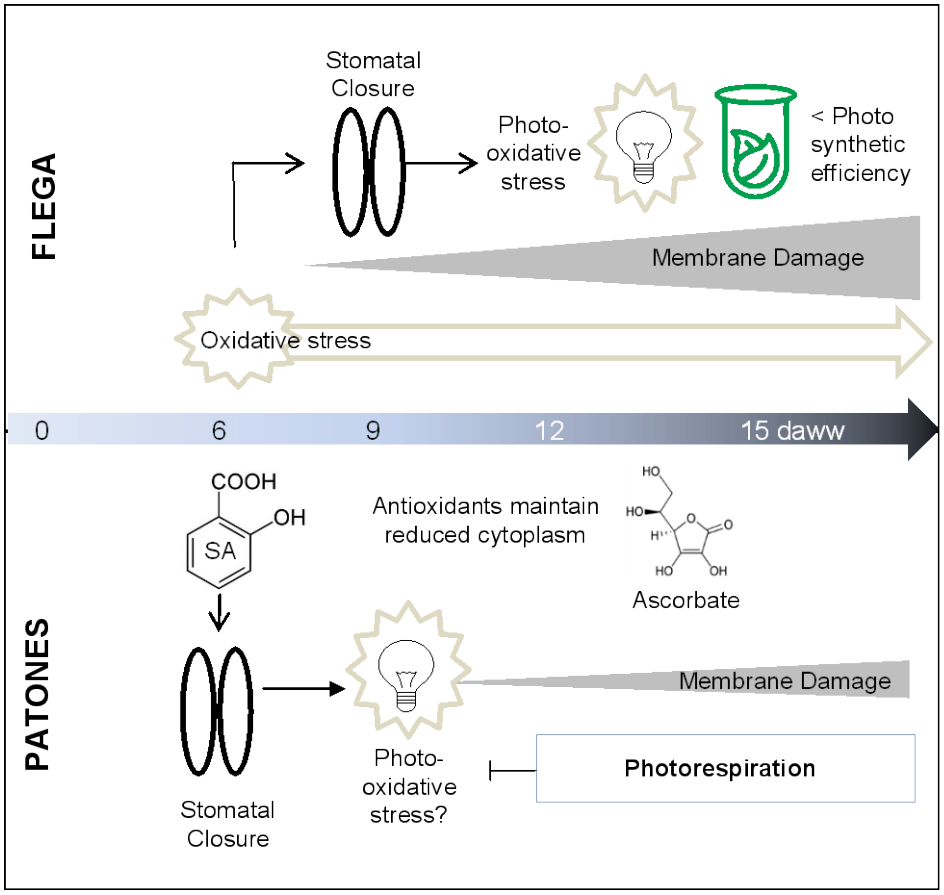
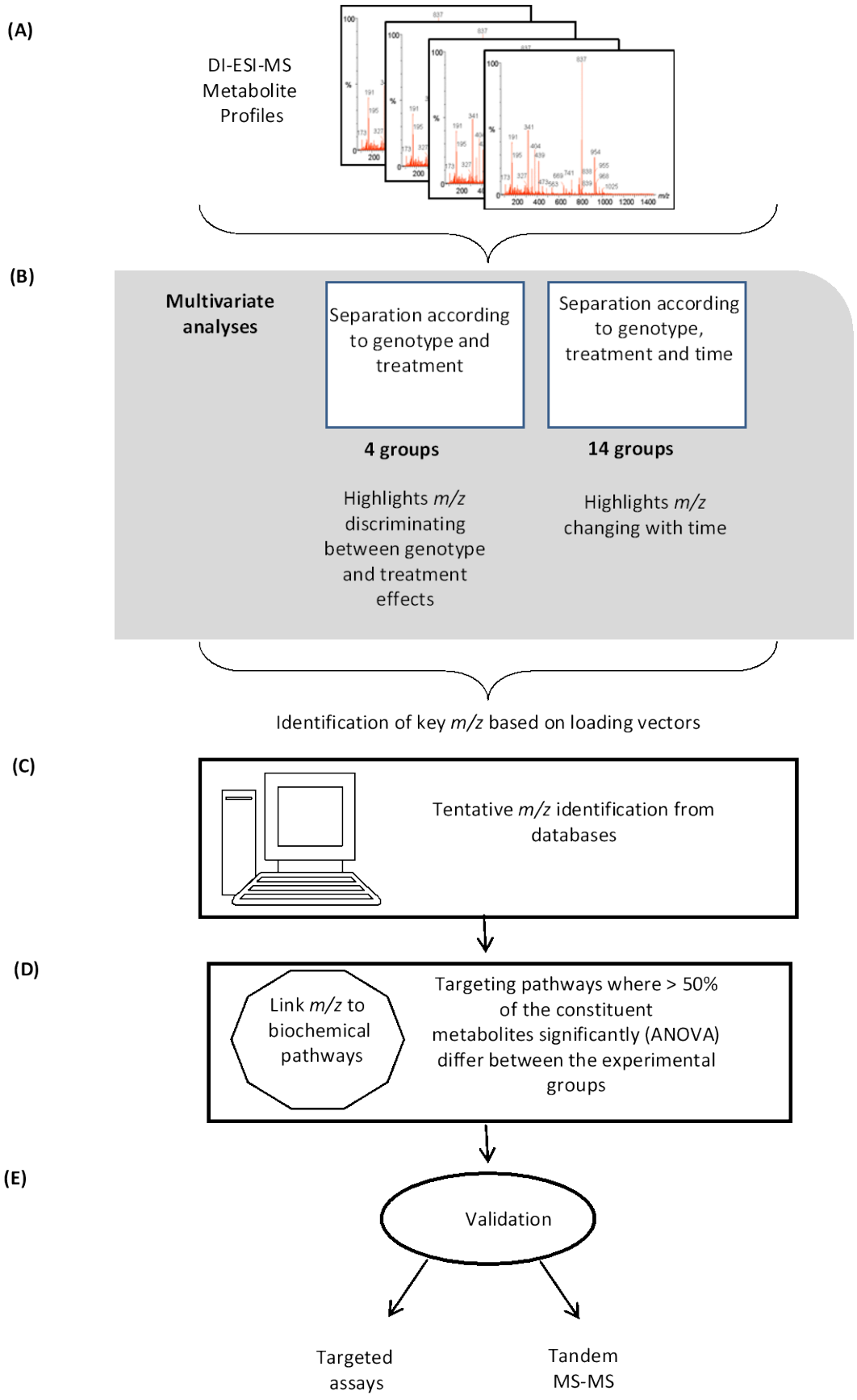

FLEGA
Stomatal Closure
Photo-oxidative stress
< Photo synthetic efficiency
Membrane Damage
Oxidative stress
0
6
9
12
15 daww
COOH
OH
SA
Antioxidants maintain reduced cytoplasm
Ascorbate
PATONES
Membrane Damage
Photo-oxidative stress?
Photorespiration
Stomatal Closure

Figure 8.

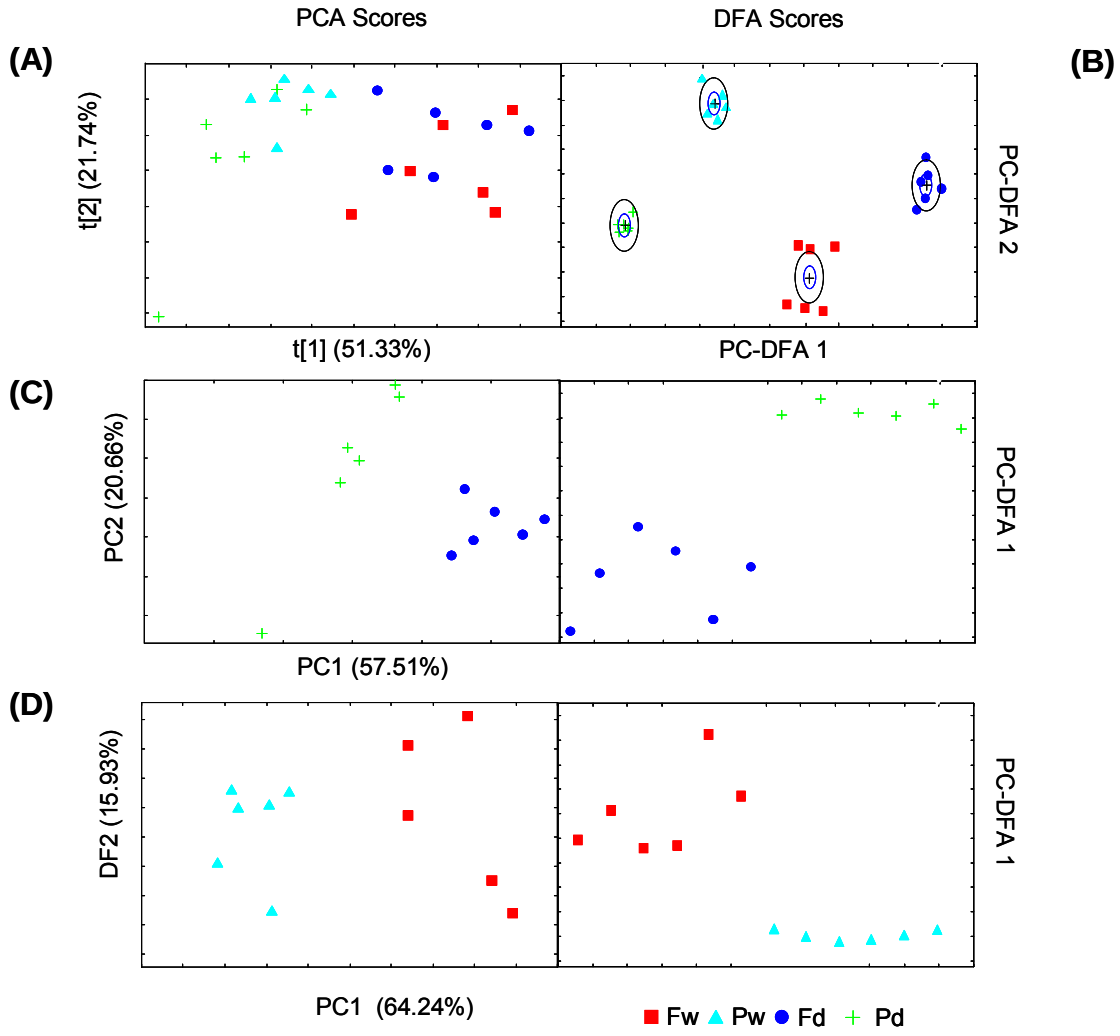
Supplementary Figure 1: Analysis Pipeline. A. Experimental samples were profiled using Direct-Infusion-Electrospray Ionisation Mass Spectroscopy (DI-ESI-MS). **B**: The multiple spectra were analysed using multivariate approaches. Data were first interrogated using Principal Component Analysis (PCA;(Causton 1987)) followed by Discriminant Function Analysis (DFA) which is a supervised projection method (Manly 1994) which attempts to between designated groups on the basis of the retained PCs. PC-DFA models were created to identify the key metabolites differentially produced in the susceptible and resistant cultivars based on deriving robust models where differences between classes were separated along a particular PC-DF axis. This allowed plotting the contributions of individual metabolites measured to the model ("loading vectors"). Those metabolites that appeared > ± 1 standard deviation (STD) from the mean value loading were recorded as significant metabolites associated with the differences. To maximise the information obtained from these analyses, PC-DFA were employed in a two-step manner 1) using classes of genotype and treatment (4 classes); 2) using classes for plants grouped according to the sampling time (14 groups). This identified the key time points and m/z to discriminate the susceptible and resistant cultivars. **C**: Each m/z was tentatively identified accord predicted true mass (m/z + 1) and interrogation ESI-MS metabolite libraries constructed from the analysis of tomato plants and olive oils (Goodacre et al., 2002) and of the Kyoto Encyclopaedia of Genes and Genomes (KEGG) metabolic pathways. **D** Predicted metabolites (based on m/z) were grouped according to the biochemical pathways and tested by ANOVA for significant differences between the experimental groups. **E**. The importance of the targeted metabolites and pathways in discriminating between the experimental classes was validated through targeted metabolite analyses and tandem MS.

Supplementary Figure 2. Example sequential analyses of Mass Spectrrometry profiles from a single experimental time point. **A** Principal Component Analysis (PCA) and **B** Discriminant Functional Analysis (DFA) of metabolite profiles from drought susceptible Flega (F) and tolerant Patones (P) droughted (d) or control well watered plants at a given time point. In B, crosses indicate mean value for each class and the circles 90 and 95% confidence intervals; **C.** DFA model based only on samples subjected to drought treatment. **D.** DFA model based on control, well watered samples. Significant metabolites explaining clustering from this three different models were compared to determine the importance of these metabolites at this specific sampling time and their constitutive or inducible condition

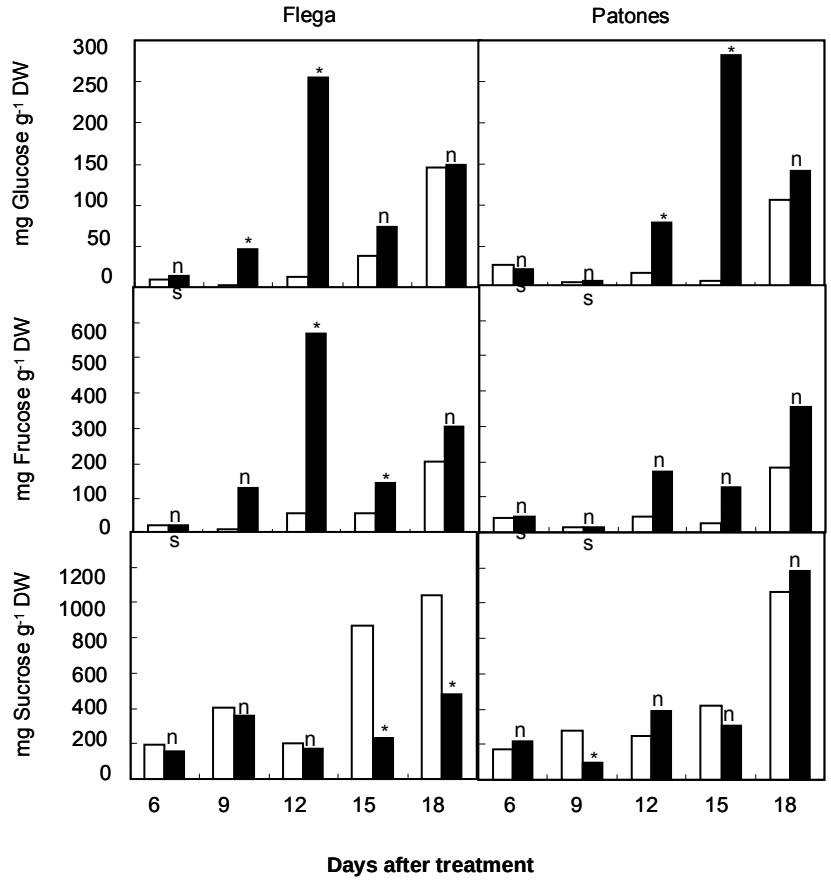
Supplementary Figure 3 . Glucose, Fructose and sucrose content in drought susceptible Flega and tolerant Patones plants during a time course of water stress (6, 9, 12 ,15 and 18 days). White bars=control, well watered plants; Black bars= plants exposed to water stress. *, **, *** indicate significant differences at $p<0.05$, 0.01 and 0.001 respectively between control and stressed plants; ns indicates no significant differences.



Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3

Identification and multi-environment validation of adaptation of *Avena sativa* cultivars to Mediterranean environments

Sánchez-Martín[1], J., Rubiales[1] D., Flores[2], F., Emeran[3], A., Shtaya[4], M.J.Y., Allagui[5], M.B., Prats[1], E.

[1]Institute for Sustainable Agriculture, CSIC, Apdo. 4084, 14080 Córdoba, Spain.
[2] E.T.S.I. La Rábida., Univ. Huelva, 21819 Palos de la Frontera, Spain
[3] Faculty of Agriculture, Kafr El-Sheikh University, 33516-Kafr El-Sheikh, Egypt.
[4]Faculty of Agriculture, An-Najah National University, P.O.Box 7, Nablus, Palestinian Territory
[5]Field crop Lab., INRAT, Rue Hédi Karray, 2049, Ariana, Tunisia

*To whom correspondence should be addressed:
Elena Prats
Institute for Sustainable Agriculture, CSIC, Apdo. 4084, E-14080 Córdoba, Spain.
E-mail: elena.prats@ias.csic.es
Tel: +34 957499291
Fax: +34 957499252

ABSTRACT

Oat (*Avena sativa* L.) ranks at around sixth in world cereal production statistics, following wheat, maize, rice, barley and sorghum. It is widely grown in temperate areas, with an increasing interest to expand the crop to subtropical areas and Mediterranean countries where drought and biotrophic fungal diseases, such as crown rust (*Puccinia coronata* f. sp. *avenae*) and powdery mildew (*Blumeria graminis* f. sp. *avenae*) seriously threaten oat production. Thus, based on a germplasm collection consisting on 141 oat landraces collected from across Southern Spain and 36 commercial cultivars, we aimed to sought and characterise new sources of resistance to both, powdery mildew and rust and drought tolerance over four crop seasons at 5 contrasting locations along Mediterranean Basin, including Spain, Egypt, and Palestinian Territories. Although the biplots revealed genotype × environment crossover interactions, the inconsistency in environment grouping did not support the concept of megaenvironments. Therefore, the Mediterranean basin oat growing region may be considered as a single complex megaenvironment. Fiel data rendered Rapidena and Alcudia could be considered as the highest yield producing cultivars but their performance was highly influenced by the environments. Patones, Primula and Caleche were the highest biomass producing cultivars being moderately stable over environments. Patones and Primula were also the highest grain yield producing and stable cultivars, but Caleche performed badly. Saia and Kankan showed the highest and most stable resistance against crown rust, followed by Alcudia. Alcudia showed the highest and most stable resistance against powdery mildew. Alcudia showed a good and stable performance for all traits evaluated in the season. Regarding resistance, our results also show that appropriate screening methods under controlled conditions may offer solid information on the resistance stability and may offer an easy and cheap mean for preliminary evaluation of segregating population.

INTRODUCTION

Oat (Avena sativa L.) is an important cereal crop cultivated over more than 9 million hectares globally (FAO, 2011). Due to its good adaptation to a wide range of soil types and because on marginal soils oats can perform better than other small-grain cereals, there is an increasing interest to expand oat cultivation crop to southern countries and even to subtropical areas(Stevens et al., 2004). Crown rust (*Puccinia coronata* f. sp. *avenae*) and powdery mildew (*Blumeria graminis* f. sp. *avenae*) are major biotic constraints for oat production. Crown rust causes high losses in yield and grain quality worldwide (Simons, 1985) but particularly in the Mediterranean basin (Herrmann and Roderick, 1996) where rust populations are more virulent than in the centre and north of Europe. Powdery mildew is of particular importance in areas with cold and humid climate (Harder and Haber, 1992). Both pathogens can be controlled with fungicides but this is expensive and harmful due to its negative effects on human health and environment. Consequently, the host resistance is being explored as the most effective, economical and environmentally friendly control method. However, the resistance obtained is often overcome by emerging pathogenic races. This is mainly caused by the inappropriate use of resistance sources, of monogenic nature. Thus, it is necessary to identify novel sources of resistance and, furthermore, the stability of sources of resistance should be evaluated through time and space, hence the importance of testing the cultivars in different localizations along the time.

Drought tolerance is most desirable in any crop, but particularly in rainfed crops in marginal areas such as oat. However, the complexity of the responses involved in the drought tolerance, including numerous physiological and molecular mechanisms, makes difficult the selection of genotypes using a single selection criterion. Besides, the selection of adequate drought tolerant genotypes in strongly affected by the Genotype by Environment interaction (GEI) (Ceccarelli, 1996). Given the difficulty of select drought tolerant genotypes, multi-environment yield trials (MEYTs) are used in the final selection cycles to identify superior genotypes in plant breeding programs. GEI attenuates the association between phenotype and genotype, reducing genetic progress in plant breeding programs. ANOVA is an additive model that describes the main effects effectively and determines if GEI is a significant source of variation, but it does not provide insight into the genotypes or environments that give rise to the interaction (Samonte et al., 2005). (Yan et al., 2000) proposed a method namely GGE-biplot, which allows visual examination of the GEI pattern of MEYT data.

The objective of this work was the identification of oat genotypes with good performance in the Mediterranean basin, with special attention to stability of resistance to crown rust and powdery mildew and drought tolerance along the different environments.

MATERIALS AND METHODS

Plant material and experimental design

An Oat Network consisting on 32 commercial varieties supplied by the Andalusian Network of Agricultural Experimentation (RAEA) was evaluated over four crop seasons at 14 contrasting locations along Mediterranean Basin, including field trials conducted in Spain, Egypt, Palestinian Territories and Tunisia. An environment was as the combination of a year and location (Table 1). The commercial varieties evaluated were: Adamo, Acebeda, Aintree, Alcudia, Anchuela, Araceli, Caleche, Cannele, Chambord, Chapline, Charming, Condor, Cory, Edelprinz, Flega, Fringante, Fuwi, Hammel, Kankan, Kantora, Karmela, Kassandra, Kazmina, Mirabel, Mojacar, Orblanche, Pallidi, Patones, Prevision, Primula, Rapidena and Saia.

Egyptian, Palestinian and Tunisian trials were performed in a single location per country over the growing seasons 2007-2008, 2008-2009 and 2009-2010. Spanish trials were performed in three contrasting locations during growing seasons 2009-2010 and 2010-2011. These environments are characterized by mild and rainy winters and warm and dry springs (Table 1), being winter sowing of spring crops a common practice. Sowings took place between October and December, according to local practices.

No artificial inoculation was performed at any location, infection occurring naturally. At each location, a randomized complete block design with three replicate was used. Each replicate consisted in independent plots consisting in three 1-m-long rows bordered by another cereal. Within each plot, the rows were separated from each other by 30 cm, at a sowing density of around 90 seeds m2.

Disease, biomass and yield assessments

Disease severity was assessed as a visual estimation of the percentage of whole plant tissue covered by pustules in the case of the crown rust and the mycelium in the case of powdery mildew. Observations were made weekly from disease onset till the end of the disease cycle. This allowed calculation of the Area Under the Disease Progress Curve (AUDPC) according to (Wilcoxson et al., 1975). To determine the data of flowering, the number of days from the sowing date until 50% anthesis was determined. At the end of the season plots were harvested and whole-plants and seeds weighted.

Statistical analysis

The GGE biplot method (Yan et al., 2000) was employed to study the genotype by location-year environment interaction of oat yield, biomass, date of flowering, AUDPC rust and AUDPC mildew. This methodology uses a biplot to show the factors (G and GE) that are important in genotype evaluation and that are also the sources of variation in GEI analysis of MEYTx data (Yan et al., 2000; Yan, 2001). The GGE/biplot shows the first 2 principal components (PC1 and PC2) derived from subjecting environment-centered trait data (trait variation due to GGE) to singular value decomposition (Yan et al., 2000; Yan, 2001). Singular value partitioning is 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 bears most of the properties associated to other scaling methods (Yan, 2002).

Cultivars and environments were represented in the same plot. This GGE biplot served as a tool to identify those cultivars widely adapted that exhibited a stable performance along all environments. Also, this could determinate the cultivars that performed well under specific conditions, and finally different mega-environments in the spring oat network could be identified. The “Average Environment Coordination” axis is the appropriate tool to compare genotypes by their average performance and stability. This axis has two components. The first component (X-axis) is the line that connects the origin and the environmental average is called as AECa, that is, the average of PC1 and PC2 coordinates across environments. The projection of each genotype onto this axis represents the effect of each genotype (G), in other words, the contribution of each genotype to G. The other component, Y-axis, AEC0 represents the contribution of each genotype to the interaction G x E, giving us the genotypic stability or instability (consistency or inconsistency along all environments). Similarly, 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).

One of the most attractive features of GGE biplot is its ability to show the "which-won-where" pattern of a genotype by environment dataset as it graphically addresses important concepts such as cross-over GE, mega-environment differentiation, specific adaptation (Yan and Tinker, 2006). The polygon view of GGE biplot explicitly displays the which-won-where pattern (Yan, 2002). The polygon is formed by connecting the markers of the genotypes that are further away from the biplot origin such that all other genotypes are contained in the polygon. The rays are lines that are perpendicular to the sides of the polygon or their extension(Yan and Kang, 2003). Genotypes located on the vertices of the polygon reveal the best or the poorest in one or other environment. A mega-environment is defined as a group of environments that consistently share the same best cultivar or cultivars. Those genotypes encompassed between two consecutive rays are taken together, as a possible mega-environment. Analyses were made by a SAS ® 9.3 (SAS Institute Inc.) program for graphing GGE biplots developed by (Burgueño et al., 2003).

RESULTS AND DISCUSSION

The analysis of variance results for spring oat data are presented in Table 2, this analysis indicated that genotype (G), environment (E) and genotype by environment (GE) interaction showed significant ($p<0.0001$) differences among oat cultivars. This result showed that all traits were significantly influenced by E which accounted for 43% to 78% of the total variation, whereas G and GE interaction explained of 2% to 14% and 5% to 27%, respectively (Table 2).

The partitioning of G + GE interaction through GGE biplot analysis showed that the first two principal components were significant factors for all traits, explaining of 67% to 99% of total G + GE interaction sum of squares (Table 2), suggesting that a biplot with the first two principal components adequately approximates the environment-centered data. Tables 3, 4, 5 and 6 showed the results for the traits evaluated; biomass, yield, powdery mildew and crown rust resistance.

Plant biomass

GGE biplot (Fig. 1) for plant biomass showed a seven-sided polygon formed by the union of the identified genotypes that were farther away from the biplot origin, so-called vertex genotypes (Cannele, Patones, Primula, Charming, Adamo, Mirabel and Condor). The projection of perpendicular lines from the biplot origin to each of these sides determined 7 sectors, some of them containing one or more environments. The vertex genotype for each sector has higher biomass than the others in all environments that fall in the sector (Yan, 2002) Thus, Cannele was the genotype with highest biomass of those falling in the first sector, defined by the vertex genotypes Condor, Cannele and Patones, grouping the environment Cord10, Esc09 and Esc10. Patones was the winner cultivar in the second sector defined by the vertex genotypes Cannele, Patones and Primula, grouping the environments Pales08, Cordo09, Sala09 and Sala10. Charming was the winner cultivar in the third sector derived from the vertex genotypes Charming and Adamo, grouping the environments Pales09 and Pales07. Primula, Adamo, Mirabel and Condor were the vertex genotypes for the fourth, fifth, sixth and seventh sectors respectively, but no environments fell into the sectors, in other words, they weren't the best in any environments. Cultivars within the polygon, particularly those located near origin, were less responsive than the vertex cultivars (Fig. 1).

The cultivars were ranked along the AEC axis abscissa (AECa), with an arrow pointing to a greater value based on their mean performance across all environments. The double arrowed line (AECo) separates entries with below –average means from those with above-

average means (in Figure 1, those genotypes that are on the left of the perpendicular have a low biomass value), and either direction away from biplot origin indicates greater genotype by environment interaction effect and reduced stability (Fig. 1). Therefore, the best genotype would be that with the highest biomass (positive projection on AECa) and the highest stability which is defined by a projection on AECo close to zero (Yan, 1999). Patones, Primula and Caleche were the cultivars producing higher (high absolute primary scores, average environment coordinate abscissa-axis) but with only a moderate stability over environments (moderate absolute secondary scores, average environment coordinate ordinate-axis). On the opposite side, Condor, Fuwi and Saia were the cultivars producing lower biomass at all environments.

In our essay we failed to identify an environment being both discriminatory and representative for this trait. Similarity, we failed to identify any mega-environment due to different localities and years were mixed in different sectors.

Grain Yield

The cultivar markers of the yield oat set (Figure 2) that was farthest from the biplot origin (cvs. Alcudia, Rapidena, Caleche, Condor, Saia and Cory) formed the corners of the polygon divided into 6 sectors. Alcudia was the winning cultivar in the first sector defined by the vertex genotypes Rapidena, Alcudia and Cory that included the environments pales08, pales09, esca09, esca10 and egip07. Rapidena was the winning cultivar for the second sector defined by the vertex cultivars Caleche, Rapidena and Alcudia including environments pales07, cordo09, cordo10, sala09, sala10, tunez08 and egip08. Caleche, Condor, Saia and Cory were the vertex genotypes for the third, fourth and fifth sectors, respectively, but no environments fell into these sectors, indicating that these genotypes were not the best in any of the environments.

Primula and Patones were the higher yielding cultivars on average and were relatively stable over the environments. In contrast, the cultivars Adamo, Mojacar, Aintree, Condor and Fringante yielded poorly at all environments. The genotype Alcudia was unstable for seed yield as it performed well at environments into first sector but poorly at the rest of environments. The genotype Rapidena was also unstable as its performance was opposite to Alcudia at the environments into first sector (Fig. 2).

Pales07, pales08 and pales09 were most discriminating (informative) environments as indicated by the largest distance between their marker projection on the average environment coordinate abscissa-axis and the origin (Fig. 2). However, due to the large secondary score on average environment coordinate ordinate-axis of the environments pales07 and pales08, cultivar differences observed at these environments may not exactly reflect the cultivar differences in average yield over all environments. Unfortunately, we failed to identify environments being both representative and discriminating that the desirable ones for selecting widely adaptative genotypes (Yan and Tinker, 2006).

The environments found in sector 1 were intentionally combined with those of sector 2 in an attempt to constitute a single mega-environment as these environments were similar to each other except the difference in yearly effects for the locations in sector 2. Therefore, no mega-environments were found.

Days to flowering

GGE biplot for days to flowering (Fig. 3) showed an eight-sided polygon formed by the union of the vertex genotypes. A double-arrow line perpendicular to AECa leaved on its right those genotypes with a longer flowering time. Genotypes Condor, Chapline, Fringante and Fuwi were characterized by a longer flowering time being consistent across environments. Genotypes Prevision, Pallidi and Kantora stand out for their short flowering time also being consistent across environments. Due to this low time of flowering, under the typically drought conditions in the Mediterranean, one might think that these genotypes could escape to this abiotic stress which reflects in an acceptable yield. If it's true that all of them showed a higher yield than the average, only Alcudia was stable across all the environments.

Their longer vectors indicated that sala10, pales07, pales08 and pales 09 were the most discriminating environments. However, none of them had small angles with AEC axis (indicative of its representation in all environments studied). The only environment with a really small angle with AECa axis was sala09, but the low value of the projection value onto the AEC abscissa, seemed to indicate that environment couldn't be used as discriminant environment. So no particular environment was more representative of the overall target environments.

Except sala10, the rest of the environments were placed in the same sector within the GGE biplot, defined by the vertex Charming and Caleche, suggesting that all Mediterranean basin is just a single mega-environment within which we could not differentiate between locations.

Response to powdery mildew

Figure 4 illustrates the GGE biplot for the AUDPC powdery mildew, derived from most extreme genotypes. The cultivar(s) with a high negative projection on AECa means that it has low disease and a projection on AEC0 close to zero is representative of high stability in the resistance.

The two-arrowed line perpendicular to AECa placed on its right those genotypes with the highest powdery mildew AUDPC and on its left those genotypes with the lowest AUDPC. Cultivar Condor showed the highest AUDPC (highest susceptibility) for powdery mildew (positive projection on AECa) being stable across environments (AECo close to zero). In a previous work (Sanchez-Martin et al., 2011) had no resistance either at seedling stage and adult plants in Adamo cultivar, which could explains the susceptibility showed by both cvs. at the field. On the contrary, cvs. Rapidena, Acebeda, Karmela, Patones, Prevision, Kankan and Kassandra showed low AUDPC values indicative of some levels of resistance (negative projection on AECa) being stable across environments (projection on AECo close to zero). A gradient of resistance may be seen within these cultivars, from the less resistant Rapidena to the most resistant Kassandra. Cv. Alcudia showed the highest level of resistance to powdery mildew (longest negative projection on AECa) but showed a slight degree of instability across environments (moderate projection on AECo).

The monogenetic sources of resistance described (Simons et al., 1978) have been traditionally overcame due to its narrow genetic resistance (Hayes and Jones, 1966) by emerging pathogenic races. Adult Plant Resistance (APR), appear to be the only effective and stable resistance in field experiments (Roderick et al., 2000). (Sanchez-Martin et al., 2011) showed that how Alcudia and Adamo reduced the macroscopic symptoms by more than 80% in the fifth leaf compared with the first leaf, indicating possible Adult Plant Resistance (APR). Microscopic assessment revealed that these cvs. showed significantly increased penetration resistance and the hypersensitive response did not add an important component to the APR in

Alcudia but it had a relative presence in the cv. Adamo. This difference could explain why the resistance in Alcudia was more stable than Adamo (long right projection on AECa) due to its well-known that prepenetration resistance is under a more complicate genetic control and it would be more complicate to be overcome by different races of the pathogen.

All the environments evaluated for this trait, were placed in the same sector within the GGE biplot, defined by the vertex Adamo and Condor, suggesting that all of them are included in a single-environment along all the Mediterranean Basin, as previously we have concluded for others traits

Response to rust

Cultivar showing lower rust AUDPC (longer negative projection on AECa) being stable across environments (lower projection on AECo) are those to be preferred. This includes cvs. Saia and Kankan, with the longest negative projection on AECa and an AECo close to zero. Interestingly, it was previously described that these two cultivars carry different mechanisms of resistance to rust (Sanchez-Martin et al., 2012). The high and stable level of resistance observed in Saia could be explained by its pre-haustorial resistance characterized by a high percentage of early aborted colonies not associated with host cell necrosis (Sanchez-Martin et al., 2012). Prehaustorial resistance plays a major role in so-called partial resistance. This is believed to be non-race-dependent and based on multiple and quantitative genes, and therefore it is more difficult to overcome by new races of pathogens than other resistance mechanisms based on single or qualitative genes, such as the HR (Niks and Rubiales, 2002). The high and stable resistance observed in Kankan, could be explained by the high percentage of pre-penetration resistance although the previous histological characterization showed us that the HR was an important component in the resistance.

Cvs. Alcudia, Primula, Chambord, Karmela and Patones showed moderate levels of resistance to the crown rust that were however not so stable across environments. In the model used here, the wider the angle between two environments in the biplot, the more divergent they are, in this case, esca10 and cord10. Alcudia showed an opposite response to Primula in these environments. This differential response might be explained by the existence of different rust races in each location, but virulence studies would be needed to conclude on this possibility.

Taking together the nature of resistance viewed at microscopically level and the resistance showed at adult plant stage, we could hypothesize that the genetic bases of resistance in Alcudia and Primula could be different, in other words, different PC resistance genes could be involved. However to make sure of that, we must do inoculations under controlled conditions with both isolated.

Esca09 was the environment with a high projection on AECa (most discriminating) and a low projection on AECo (most representative) for rust resistance.All the environments but esca10 were placed in the same sector within the GGE biplot, defined by the vertex Charming and Caleche, suggesting all Mediterranean basin is just a single mega-environment within which we could not differentiate between locations.

Availability of cultivars with good yield potential and resistance to major biotic and abiotic stresses is needed for sustainable oat crop production. In this study we compared stability in time and space of yield and powdery mildew and rust incidence in a collection of oat cultivars tested across several crops seasons and countries. Each trait analyzed separately, showed the different responses of the accessions. However, as the variations in ranking show, it is necessary to exam the stability of the material and its interaction with the environment.

The really high value (from 43-78 % of total sum of squares) of the variation due to Environment (E), which is irrelevant to cultivar evaluation and mega-environment (Fox and Rosielle, 1982; Gauch and Zobel, 1997) demonstrated that the environments were different and contrasting, with important variations in their average values. All of this, justifies the uses of GGE biplot method to analyze the data form multi-environment trials (Crossa and Cornelius, 1997).

Although the biplots revealed genotype × environment crossover interactions, the inconsistency in environment grouping did not support the concept of megaenvironments. Therefore, the Mediterranean basin oat growing region may be considered as a single complex megaenvironment, where unpredictable crossover GE interactions are expected.

Rapidena and Alcudia could be considered as the highest yield producing cultivars but their performance was highly influenced by the environments. Patones, Primula and Caleche were the highest biomass producing cultivars being moderately stable over environments. Patones and Primula were also the highest grain yield producing and stable cultivars, but Caleche performed badly. Saia and Kankan showed the highest and most stable resistance against crown rust, followed by Alcudia. Alcudia showed the highest and most stable resistance against powdery mildew. Alcudia showed a good and stable performance for all traits evaluated in the season.

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TABLES

Table 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.

Environm.	Location	Latit.	Long.	Altitude (m ASL)	Growing season	Weather during growing season		
						Absolute Max. T (°C)	Absolute Min. T (°C)	Rain (mm)
Cordo09	Córdoba, Spain	37° 50' 39" N	4° 50' 46" W	90	2009-2010	31,7	-2,1	723
Cordo10	Córdoba, Spain	37° 50' 39" N	4° 50' 46" W	90	2010-2011	33,6	0,2	278
Egip07	Kafr El-Sheik, Egypt	28° 28' 0" N	30° 49' 60" W	8	2007-2008	47.8	-6.1	40
Egip08	Kafr El-Sheik, Egypt	28° 28' 0" N	30° 49' 60" W	8	2008-2009	48.5	-4.3	37
Esca09	Escacena, Spain	37° 25' 22" N	6° 15' 13" W	88	2009-2010	34.8	0.0	392
Esca10	Escacena, Spain	37° 25' 22" N	6° 15' 13" W	88	2010-2011	33.4	-0.3	357
Pales07	Nablus, Palest. Territ.	32° 31' 51,9 " N	35° 02' 0,33" W	75	2007-2008	27,2	12,4	448
Pales08	Nablus, Palest. Territ.	32° 31' 51,9 " N	35° 02' 0,33" W	75	2008-2009	25,1	11,2	437
Pales09	Nablus, Palest. Territ.	32° 31' 51,9 " N	35° 02' 0,33" W	75	2009-2010	25,8	12,7	412
Sala09	Salamanca, Spain	40° 55' 28.2 " N	5° 21' 45.54 " W	829	2009-2010	31,1	-5,8	296
Sala10	Salamanca, Spain	40° 55' 28.2 " N	5° 21' 45.54 " W	829	2010-2011	37,2	-7,5	253
Tunez07	Beja, Tunisia	36° 43' 59 " N	9° 10' 59" E	222	2007-2008	42.1	-11.7	430
Tunez08	Beja, Tunisia	36° 43' 59 " N	9° 10' 59" E	222	2008-2009	43.3	-11.2	413
Tunez09	Beja, Tunisia	36° 43' 59 " N	9° 10' 59" E	222	2009-2010	44.5	-10.8	420

Table 2. Genotype (G), location-year environment (E) and genotype by location-year environment interaction (GE) terms for yield (kilograms per hectare), Biomass, date of flowering, AUDPC rust and AUDPC mildew for the spring oat performance trials, from 2007 to 2010.

Trait	Source of variation	df[a]	Mean Squares[b]	Explained variation % of G, E and GE[c]	% of PC1 + PC2[d]
Yield	E	11	360627994***	77	61+18
	G	31	6703685***	4	
	GE	341	1528952***	10	
Biomass	E	8	44.92***	78	48+19
	G	31	0.34***	2	
	GE	248	0.13***	7	
Date of flowering	E	9	54700***	78	80+9
	G	31	2375***	12	
	GE	279	115***	5	
AUDPCrust	E	13	188.91***	43	70+8
	G	31	24.88***	14	
	GE	403	3.78***	27	
AUDPCmildew	E	4	341.85***	60	88+11
	G	31	5.97***	8	
	GE	124	4.35***	24	

[a] degrees of freedom

[b] *** Significant at the 0.0001 level of probability.

[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.

Table 3 Biomass of 32 cultivars of *Avena sativa* at 14 location-year environments from Spring Oat Network.

Cultivar	cordo09	cordo10	esca09	esca10	pales07	pales08	pales09	sala09	sala10	Total
Acebeda	0,34	0,29	0,33	0,53	25,45	0,51	16,90	0,41	0,65	0,81
Adamo	0,42	0,26	0,21	0,39	26,37	0,39	18,17	0,24	0,38	0,75
Aintree	0,41	0,23	0,22	0,76	19,72	0,34	13,60	0,37	0,81	0,72
Alcudia	0,79	0,37	0,69	0,75	22,30	0,81	15,82	0,39	0,59	0,91
Anchuela	0,81	0,23	0,78	0,73	27,32	0,44	17,00	0,27	0,72	0,93
Araceli	0,58	0,31	0,30	0,76	23,90	0,61	10,02	0,55	0,55	0,78
Caleche	0,52	0,29	0,18	0,78	32,42	0,30	16,78	0,46	14,00	0,98
Cannele	0,82	0,54	0,45	10,75	15,38	0,57	12,30	0,56	0,83	0,85
Chambord	0,91	0,37	0,62	0,98	21,93	0,55	17,15	0,32	0,61	0,92
Chappline	0,58	0,29	0,28	0,68	21,65	0,37	14,02	0,53	10,03	0,81
Charming	0,60	0,38	0,19	0,61	31,92	0,47	18,23	0,78	0,93	1,00
Condor	0,73	0,13	0,39	0,39	21,15	0,32	11,38	0,25	0,43	0,66
Corry	0,69	0,32	0,39	0,71	30,10	0,78	19,19	0,38	0,70	0,99
Edelprinz	0,82	0,32	0,40	0,78	25,57	0,69	16,88	0,51	0,75	0,95
Flega	0,49	0,33	0,44	0,70	22,22	0,71	14,53	0,42	0,49	0,81
Frigante	0,78	0,35	0,30	0,71	25,73	0,40	15,45	0,71	0,82	0,91
Fuwi	0,48	0,23	0,13	0,66	22,52	0,21	14,11	0,43	0,47	0,70
Hamel	0,74	0,45	0,33	0,93	22,70	0,54	16,31	0,28	0,65	0,87
Kankan	0,56	0,32	0,63	10,08	18,72	0,60	12,73	0,43	10,06	0,86
Kantora	0,80	0,39	0,48	0,90	20,18	0,58	13,11	0,55	0,59	0,84
Karmela	0,60	0,33	0,43	0,66	22,38	0,59	14,21	0,57	0,86	0,86
Kassandra	0,87	0,35	0,43	0,89	22,15	0,40	12,03	0,50	0,58	0,84
Kazmina	0,62	0,31	0,37	0,51	19,47	0,71	14,08	0,45	11,04	0,83
Mirabel	0,57	0,14	0,21	0,22	23,97	0,65	15,62	0,29	0,33	0,71
Mojacar	0,51	0,29	0,31	0,54	25,90	0,19	12,96	0,22	0,52	0,72
Orblanche	0,84	0,40	0,36	0,60	23,92	0,66	16,84	0,43	0,63	0,89
Pallidi	0,71	0,41	0,38	0,62	22,90	0,46	15,70	0,79	0,76	0,89
Patones	0,66	0,34	0,34	0,89	26,88	0,59	17,48	0,54	14,26	10,24
Prevision	0,64	0,27	0,51	0,54	22,97	0,18	16,11	0,45	0,69	0,80
Primula	0,96	0,34	0,50	0,68	31,88	0,77	22,08	0,60	11,78	11,58
Rapidena	11726,00	0,35	0,41	0,66	30,97	0,62	18,06	0,51	0,72	10,38
Saia	0,66	0,38	0,33	0,63	20,30	0,49	16,73	0,13	0,41	0,75
Total	0,68	0,32	0,38	0,69	24091,00	0,52	15,49	0,45	0,74	0,86
SE	0,02	0,01	0,02	0,03	0,07	0,03	0,04	0,02	0,04	0,02

Table 4 Mean Grain Yield (Kg per hectare) standard error (SE) of 32 cultivars of Avena sativa at 14 location-year environments from Oat Network.

Accession	cord09	cord10	egip07	egip08	esc09	esc10	pales07	pales08	pales09	sala09	sala10	tunez08	Total
Acebeda	289,8	340,7	1760,0	2261,7	25,0	1088,9	8888,9	1530,8	4759,3	117,7	770,4	604,8	1869,8
Adamo	535,7	307,4	1046,7	1563,3	25,6	440,7	4629,6	598,3	3385,2	112,5	468,9	600,7	1142,9
Aintree	299,3	318,5	713,3	1130,0	19,9	1763,0	4685,2	413,3	3774,1	102,4	2186,4	653,0	1338,2
Alcudia	459,7	463,0	2763,3	2556,7	114,9	1581,5	8370,4	4975,0	6264,8	120,8	1381,3	443,0	2457,9
Anchuela	485,4	274,1	2306,7	2280,0	144,0	1311,1	7814,8	932,0	5913,0	88,9	2251,8	689,6	2040,9
Araceli	346,5	425,9	1733,3	1826,7	14,8	1648,1	7796,3	1796,3	3644,4	224,3	998,6	696,3	1762,6
Caleche	354,2	611,1	706,7	1690,0	18,1	1255,6	7722,2	394,9	3681,5	326,1	2175,0	683,3	1634,9
Cannele	599,4	1048,1	1426,7	1573,3	62,5	2544,4	5666,7	1610,8	3366,7	274,3	2521,3	1025,9	1810
Chambord	665,0	300,0	2406,7	2286,7	82,1	2096,3	7981,5	2444,4	5255,6	78,9	1329,6	949,3	2156,3
Chappline	406,6	537,0	793,3	1283,3	20,0	1637,0	5463,0	1314,6	4755,6	153,3	2631,9	607,8	1633,6
Charming	546,1	570,4	1113,3	1666,7	14,7	1070,4	6925,9	573,9	3950,0	292,7	2601,1	560,7	1657,2
Condor	590,2	148,2	1140,0	901,7	34,1	566,7	3092,6	407,2	3098,1	61,8	514,6	744,4	941,63
Corry	423,6	485,2	1366,7	1303,3	46,5	1211,1	4333,3	4024,5	5014,8	170,7	1214,6	758,2	1696
Edelprinz	485,1	529,6	1603,3	1780,0	39,9	1681,5	5870,4	2999,8	4561,1	129,1	1451,6	837,8	1830,8
Flega	445,8	792,6	2343,3	2340,0	51,7	1800,0	7240,7	3074,1	5051,9	120,3	754,1	354,8	2030,8
Frigante	593,3	577,8	936,7	1400,0	46,7	1570,4	3422,2	1092,4	2883,3	249,8	1351,4	771,1	1241,3
Fuwi	519,8	166,7	950,0	1690,0	50,7	703,7	6222,2	240,7	3950,0	419,6	762,9	231,9	1321,9
Hamel	514,4	696,3	1943,3	2176,7	32,2	2059,3	7463,0	2611,1	5274,1	121,2	1288,9	607,0	2065,6
Kankan	511,7	422,2	2033,3	2073,3	119,4	2811,1	6722,2	2759,0	4737,0	211,3	2929,6	306,7	2244
Kantora	623,3	559,3	2043,3	2286,7	62,6	2151,9	7407,4	2963,0	5440,7	221,2	1796,3	492,6	2170,7
Karmela	551,4	488,9	493,3	1960,0	45,6	770,4	7870,4	3018,5	5079,6	211,5	1863,0	1761,9	2009,5
Kassandra	808,2	703,7	1590,0	2290,0	75,8	2148,1	8203,7	1049,3	4625,9	182,6	1548,1	868,2	2060
Kazmina	442,3	570,4	2053,3	2185,0	23,8	1714,8	7000,0	3826,9	4727,8	267,9	2037,0	384,8	2102,8
Mirabel	499,0	448,2	666,7	1346,7	20,0	344,4	5166,7	3005,9	3931,5	66,6	685,1	483,3	1388,7
Mojacar	403,9	300,0	1296,7	1503,3	50,5	1159,3	4833,3	472,4	4005,6	125,0	976,4	516,7	1303,6
Orblanche	562,0	588,9	1970,0	1556,7	34,8	1081,5	4666,7	1821,0	3459,3	155,6	1560,4	888,2	1528,7
Pallidi	676,0	822,2	1633,3	2320,0	118,7	1537,0	8222,2	2222,0	6070,4	382,6	2470,2	585,2	2255
Patones	440,1	466,7	1386,7	2340,0	18,2	2192,6	8314,8	2888,9	5505,6	195,9	4322,2	520,0	2382,6
Prevision	441,6	470,4	1543,3	2120,0	70,2	1018,5	7537,0	296,2	4913,0	153,0	758,0	1031,5	1696
Primula	916,2	463,0	2063,3	2493,3	47,4	1281,5	8500,0	3055,6	6292,6	212,4	3645,7	1048,1	2501,6
Rapidena	748,1	670,4	1933,3	2800,0	65,5	1603,7	9888,9	2703,7	6607,4	134,1	1940,7	1480,4	2548
Saia	589,2	62963,0	1150,0	1050,0	15,6	574,1	2814,8	1592,6	4394,4	42,8	547,9	215,2	1087,5
Total	524,2	488,4	1528,4	1876,1	48938,0	1450,6	6585,5	1959,7	4636,7	178,9	1679,2	708,4	1808,7
SE	18222,0	25695,0	66566,0	61830,0	36710,0	79858,0	229,9	143,9	156,0	10891,0	105,4	42415,0	62331

Table 5 Mean AUDPC Powdery Mildew and standard error (SE) of 32 cultivars of *Avena sativa* at 14 location-year environments from Oat Network.

Accession	cordo09	cordo10	egip07	esca09	esca10	pales07	pales08	pales09	sala09	sala10	Total
Acebeda	146,7	84	147,7	164,0	121,3	129,0	119,7	112,7	162,3	163,0	135,0
Adamo	165,0	112,0	151,0	174,0	151,3	152,0	133,0	133,7	170,7	186,0	152,9
Aintree	168,3	107,0	153,0	170,7	146,0	172,0	159,0	151,0	176,7	181,0	158,5
Alcudia	146,7	80,0	139,3	164,0	116,0	129,0	113,0	113,0	168,3	172,0	134,1
Anchuela	150,0	92,7	144,3	164,0	129,0	130,3	115,0	114,0	168,3	186,0	139,4
Araceli	160,0	97,0	143,7	164,0	139,0	143,7	134,7	125,0	170,7	163,0	144,1
Caleche	168,3	112,0	153,3	167,3	154,0	172,0	155,0	154,3	174,3	186,0	159,7
Cannele	165,0	97,0	154,0	164,0	139,0	151,3	137,0	129,0	168,3	186,0	149,1
Chambord	150,0	97,0	134,7	164,0	134,0	135,7	124,0	117,0	173,0	181,0	141,0
Chappline	168,3	107,0	141,3	174,0	146,0	164,0	141,3	144,7	180,3	186,0	155,3
Charming	171,7	112,0	162,0	173,3	151,3	172,0	156,7	156,7	180,3	186,0	162,2
Condor	168,3	112,0	153,0	174,0	146,3	161,0	143,3	142,0	180,3	186,0	156,6
Corry	165,0	112,0	147,7	174,0	146,0	152,0	126,3	133,3	168,3	186,0	151,1
Edelprinz	150,0	102,0	157,7	170,7	143,7	138,0	124,7	124,0	170,7	185,3	146,7
Flega	146,7	84,0	130,3	167,3	118,7	129,0	111,0	112,7	155,0	172,0	132,7
Frigante	168,3	112,0	154,7	170,7	146,0	164,0	144,0	144,7	170,7	186,0	156,1
Fuwi	171,7	112,0	154,0	167,3	146,0	164,0	146,0	139,7	178,0	186,0	156,5
Hamel	150,0	92,7	144,3	164,0	134,0	142,0	128,0	126,0	168,3	186,0	143,6
Kankan	150,0	84,0	138,7	164,0	124,0	138,0	125,3	120,0	168,3	159,0	136,2
Kantora	150,0	84,0	141,7	164,0	139,0	138,0	129,0	121,0	170,7	172,0	141,1
Karmela	143,3	84,0	144,3	167,3	124,0	129,0	113,7	112,0	162,3	159,0	133,9
Kassandra	150,0	84,0	153,7	164,0	124,0	149,0	126,0	132,0	166,0	163,0	140,3
Kazmina	150,0	84,0	145,7	164,0	121,3	130,0	116,3	113,3	166,0	148,0	133,9
Mirabel	171,7	112,0	147,3	176,7	146,3	172,0	151,0	138,0	178,0	176,0	156,4
Mojacar	165,0	107,0	151,3	170,7	136,3	151,3	131,7	133,3	178,0	177,0	150,2
Orblanche	165,0	102,7	148,7	167,3	146,0	144,7	136,3	141,3	176,7	186,0	151,5
Pallidi	150,0	84,0	146,3	164,0	136,3	136,3	121,3	122,3	174,3	172,0	140,7
Patones	150,0	84,0	137,7	164,0	124,0	153,7	119,0	109,0	168,3	159,0	136,9
Prevision	150,0	84,0	140,0	164,0	136,3	139,0	122,3	120,3	170,7	171,0	139,8
Primula	155,0	102,0	148,0	164,0	141,3	142,0	129,3	124,3	174,3	186,0	146,6
Rapidena	150,0	88,3	141,7	164,0	124,0	129,0	114,3	111,3	164,7	172,0	135,9
Saia	150,0	82,7	154,0	164,0	134,0	145,0	129,3	120,0	164,7	167,0	142,0
Total	157,2	96,9	147,0	167,3	136,4	146,8	130,5	127,9	170,9	176,0	145,6
SE	10,1	12,7	11,1	0,5	11,9	15,6	15,6	14,7	0,9	12,8	0,8

Table 6 Mean AUDPC rust and standard error (SE) of 32 cultivars of *Avena sativa* at 14 location-year environments from Oat Network.

Accession	cordo09	cordo10	egip07	egip08	esca09	esca10	pales07	pales08	pales09	sala09	sala10	tunez07	tunez08	tunez09	Sev. mean
Acebeda	0,26	59,03	16,67	14,39	47,23	14,53	0,01	13,54	16,67	0,26	0,11	0,01	0,01	49,29	17,00
Adamo	0,77	58,19	11,11	12,88	88,14	39,28	12,89	22,49	12,22	12,02	0,42	0,06	0,06	65,36	24,83
Aintree	0,29	53,26	18,06	18,18	55,82	10,79	0,50	22,71	0,64	0,31	0,94	0,01	0,01	33,98	17,14
Alcudia	0,13	0,18	0,07	0,30	16,71	0,00	0,00	0,00	0,56	0,96	0,02	0,00	0,00	14,10	0,38
Anchuela	0,14	0,32	0,15	0,45	15,13	0,05	0,00	0,30	11,78	10,83	0,47	0,01	0,01	0,40	0,44
Araceli	0,33	80,49	0,49	0,92	78,20	38,16	0,27	15,52	10,89	0,49	52,78	0,00	0,00	96,71	29,69
Caleche	18,44	47,85	12,50	12,88	62,12	36,59	10,00	21,50	0,68	10,71	27,22	0,02	0,02	23,69	20,77
Cannele	0,29	34,31	0,39	0,61	38,03	17,42	0,78	0,56	0,86	11,91	0,02	0,04	0,04	25,89	11,66
Chambord	0,02	0,28	0,38	0,77	19,84	10,05	0,00	0,12	17,22	19,05	0,00	0,00	0,00	15,75	0,70
Chappline	0,98	59,93	22,22	23,49	67,24	0,50	0,78	10,40	0,88	0,45	0,97	0,01	0,01	54,21	20,24
Charming	0,75	75,76	10,56	11,36	74,78	14,10	0,94	0,90	0,91	0,11	13,07	0,05	0,05	29,72	19,03
Condor	0,57	56,04	27,08	28,79	56,75	62,99	11,67	27,45	10,00	0,33	0,37	0,04	0,04	43,23	24,10
Corry	11,76	54,17	0,15	15,91	78,83	64,83	0,67	0,24	10,67	0,85	23,94	0,01	0,01	60,04	24,25
Edelprinz	0,77	40,63	22,22	28,03	50,26	44,10	0,11	12,55	12,33	11,67	0,55	0,02	0,02	51,01	20,76
Flega	0,36	55,35	0,43	0,26	79,69	26,15	0,39	0,90	12,89	11,55	17,22	0,01	0,01	33,55	18,57
Frigante	0,88	65,49	36,11	37,12	68,04	25,90	0,11	13,14	0,86	10,71	22,22	0,10	0,10	47,02	24,72
Fuwi	0,83	45,49	31,94	33,33	73,89	26,24	11,22	21,49	0,50	0,45	0,61	0,02	0,02	48,29	22,59
Hamel	0,10	23,19	0,11	0,26	24,10	0,63	0,00	0,72	0,87	10,83	16,57	0,01	0,01	24,22	0,90
Kankan	0,05	0,00	0,79	10,61	0,07	0,27	0,02	0,89	0,69	0,05	0,06	0,00	0,00	0,00	0,32
Kantora	0,10	24,17	0,04	0,17	36,42	14,53	0,00	0,02	10,22	0,38	0,30	0,00	0,00	24,02	0,84
Karmela	0,23	49,44	0,56	0,53	32,64	0,67	0,00	11,94	0,82	0,60	0,94	0,02	0,02	0,02	0,99
Kassandra	0,05	48,26	14,58	14,39	47,45	10,06	0,03	0,84	0,87	0,13	0,06	0,05	0,05	28,94	13,46
Kazmina	0,13	27,61	0,10	0,30	21,76	16,00	0,00	1,19	0,76	0,50	0,06	0,00	0,00	0,66	0,65
Mirabel	0,46	38,96	0,90	0,32	86,91	32,61	0,89	17,88	0,41	15,71	20,35	0,08	0,08	52,98	21,21
Mojacar	0,43	22,08	10,42	11,36	54,50	39,41	0,50	0,30	11,22	12,02	18,89	0,08	0,08	35,12	16,36
Orblanche	0,28	33,13	11,81	0,45	40,90	15,83	0,23	10,19	20,56	11,43	20,33	0,02	0,02	41,83	15,44
Pallidi	0,10	10,25	0,15	0,23	18,09	0,48	0,00	0,24	0,74	0,74	22,87	0,00	0,00	12,41	0,65
Patones	10,31	0,00	0,14	0,68	59,72	11,57	0,03	11,33	0,56	0,80	0,11	0,00	0,00	0,41	0,86
Prevision	0,00	14,44	0,00	0,08	0,69	0,62	0,07	0,93	0,64	0,21	0,11	0,02	0,02	23,03	0,51
Primula	0,07	28,54	0,32	0,30	24,80	0,18	0,00	0,06	11,33	0,80	0,17	0,00	0,00	0,74	0,65
Rapidena	11,01	50,07	0,86	10,61	76,84	22,18	0,19	11,62	15,00	11,07	22,04	0,00	0,00	32,75	19,55
Saia	0,00	0,35	0,21	0,08	0,00	0,00	0,00	0,24	12,78	0,20	0,00	0,00	0,00	0,13	0,18
Sev. mean	0,46	36,48	0,96	10,95	47,44	19,66	0,35	0,99	0,99	0,77	10,65	0,02	0,02	31,38	14,46
SE	0,06	0,26	0,12	0,11	0,30	0,22	0,07	0,09	0,05	0,07	0,18	4,04	4,00	0,25	0,06

FIGURE LEGENDS

Figure 1. GGE biplot based on plant biomass of 32 spring oat cultivars grown at 9 location-year environments, from 2007 to 2010. 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.

Figure 2. GGE biplot based on the grain yield data of 32 spring oat cultivars grown at 12 location-year environments, from 2007 to 2010. 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.

Figure 3. GGE biplot based on the days to flowering of 32 spring oat cultivars grown at 10 location-year environments, from 2007 to 2010. 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.

Figure 4. GGE biplot based on the AUDPC powery mildew data of 32 oat cultivars grown at 5 location-year environments, from 2007 to 2009. 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.

Figure 5. GGE biplot based on the AUDPC rust data of 32 spring oat cultivars grown at 14 location-year environments, from 2007 to 2010. 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.

FIGURES

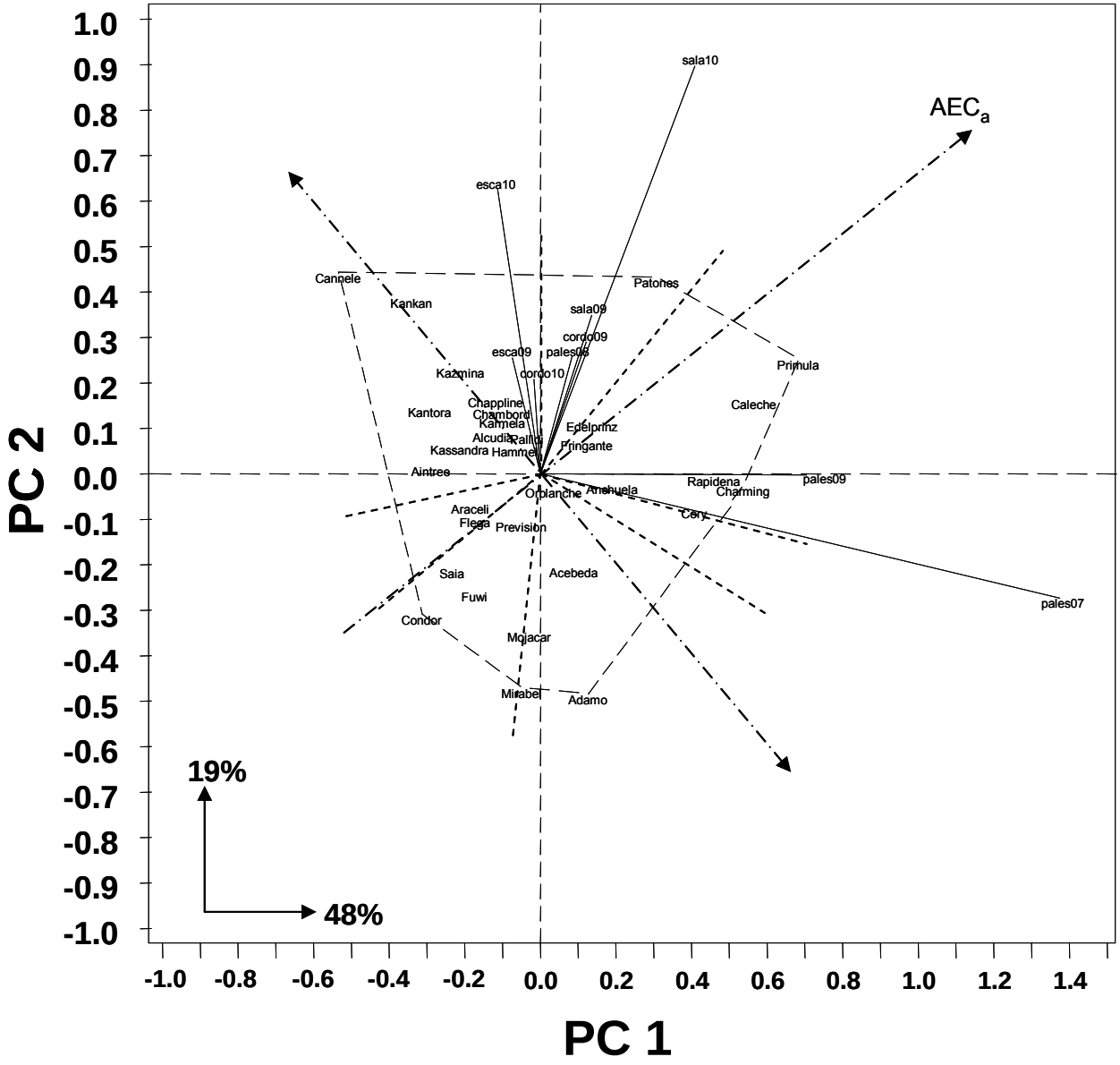

Figure 1

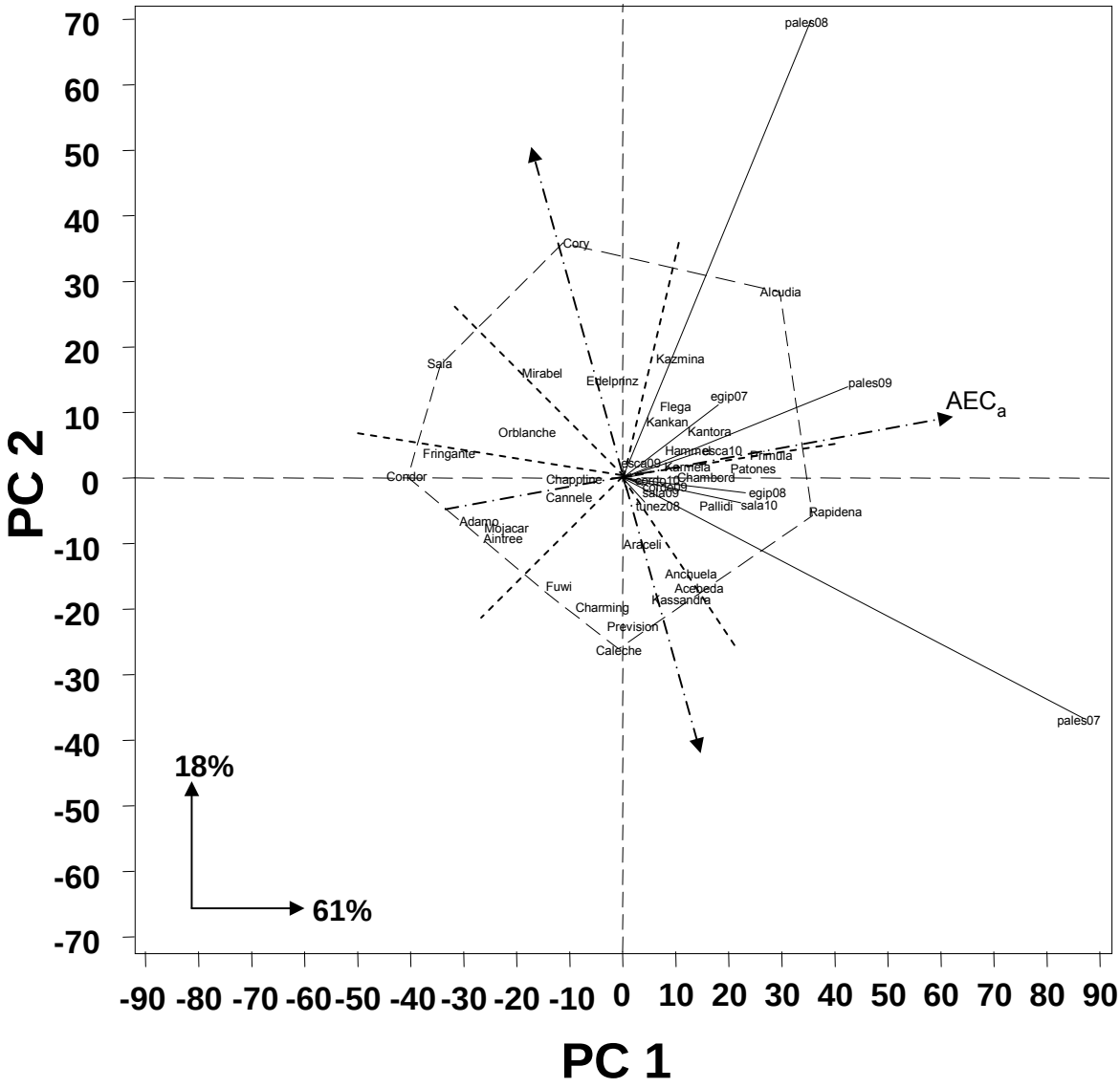
PC 2
PC 1
18%
61%
AEC$_a$
pales08
pales09
pales07
Cory
Alcudia
Sara
Mirabel
Eldelprinz
Kazmina
egip07
Flega
Kankan
Kantora
Orblanche
Fringante
Hammiesca10
Primula
Condor
Chappline
Chambord
Patones
Cannele
egip08
sala10
Pallidi
Rapidena
Adamo
Mojacar
Aintree
Araceli
Anchuela
Acebeda
Fuwi
Charming
Kassandra
Prevision
Caleche

Figure 2.

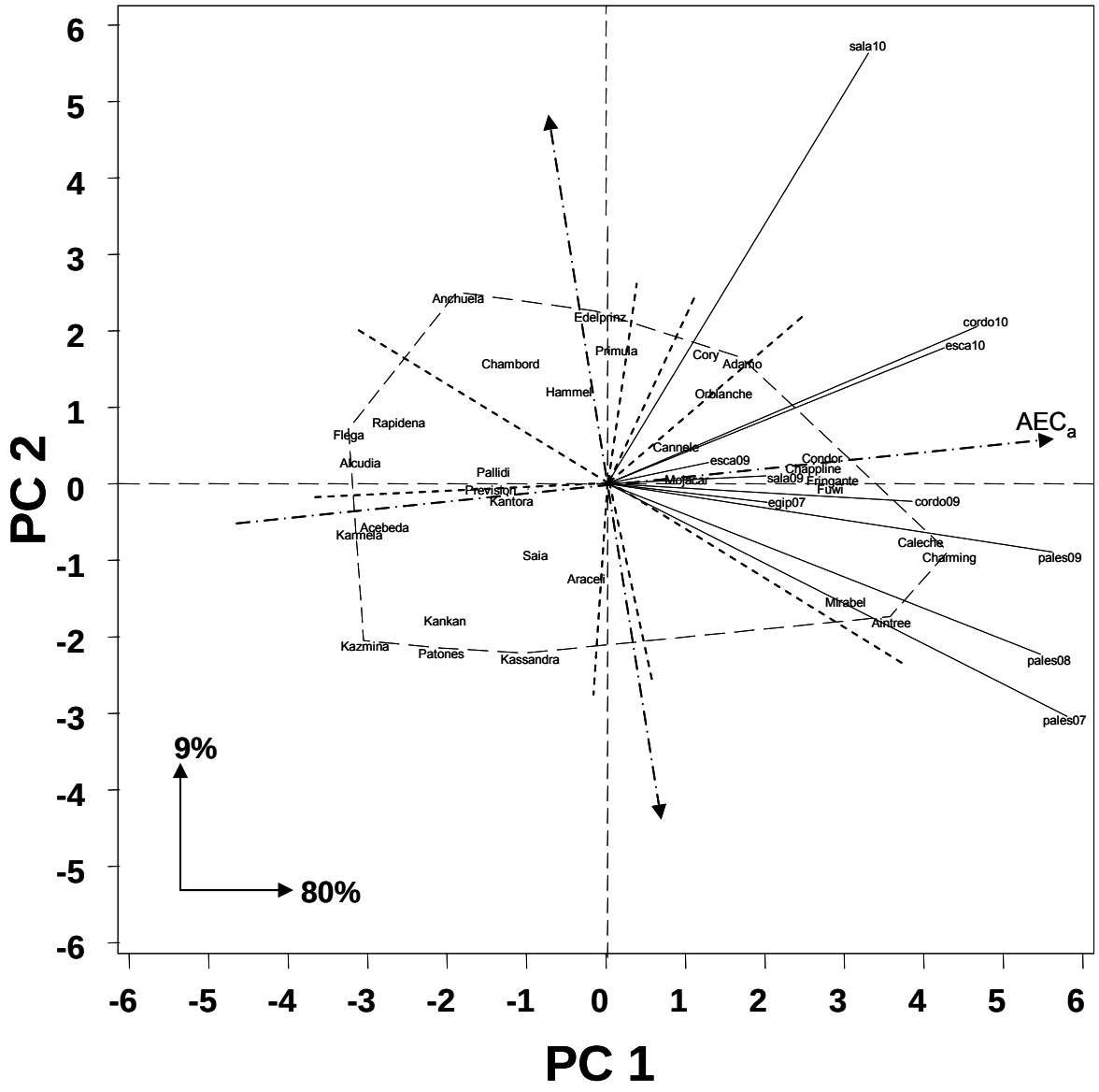
PC 2
PC 1
AEC$_a$
9%
80%
sala10
Anzhuela
Edelprinz
Primula
Cory
Adamo
cordo10
esca10
Chambord
Hammel
Orblanche
Rapidena
Flega
Alcudia
Cannele
esca09
Condor
Chappline
Pallidi
Previstori
Kantora
Mojacar
sala09
Fringante
Fuwi
egip07
cordo09
Acebeda
Karmela
Caleche
Charming
pales09
Saia
Araceli
Mirabel
Kankan
Aintree
Kazmina
Patones
Kassandra
pales08
pales07

Figure 3.

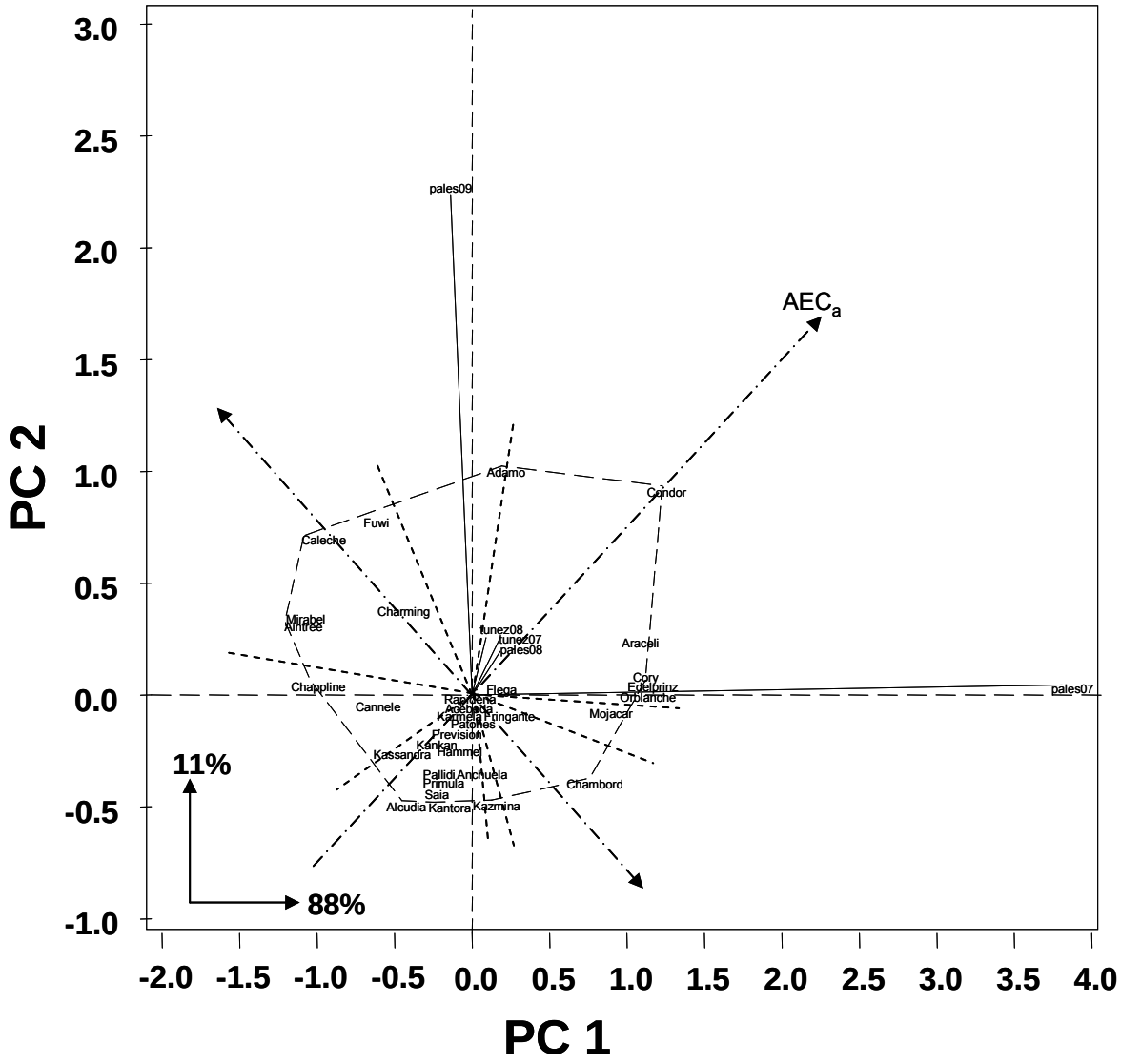
PC 2
PC 1
AEC$_a$
11%
88%
pales09
Adamo
Condor
Fuwi
Caleche
Charming
Mirabel
Araceli
funez08
funez07
pales08
Chappline
Cannele
Flega
Cory
Edelbrinz
Oblanche
pales07
Mojacar
Kassandra
Hammel
Kankan
Chambord
Pallidi
Primula
Saia
Alcudia
Kantora
Kazmina

Figure 4.

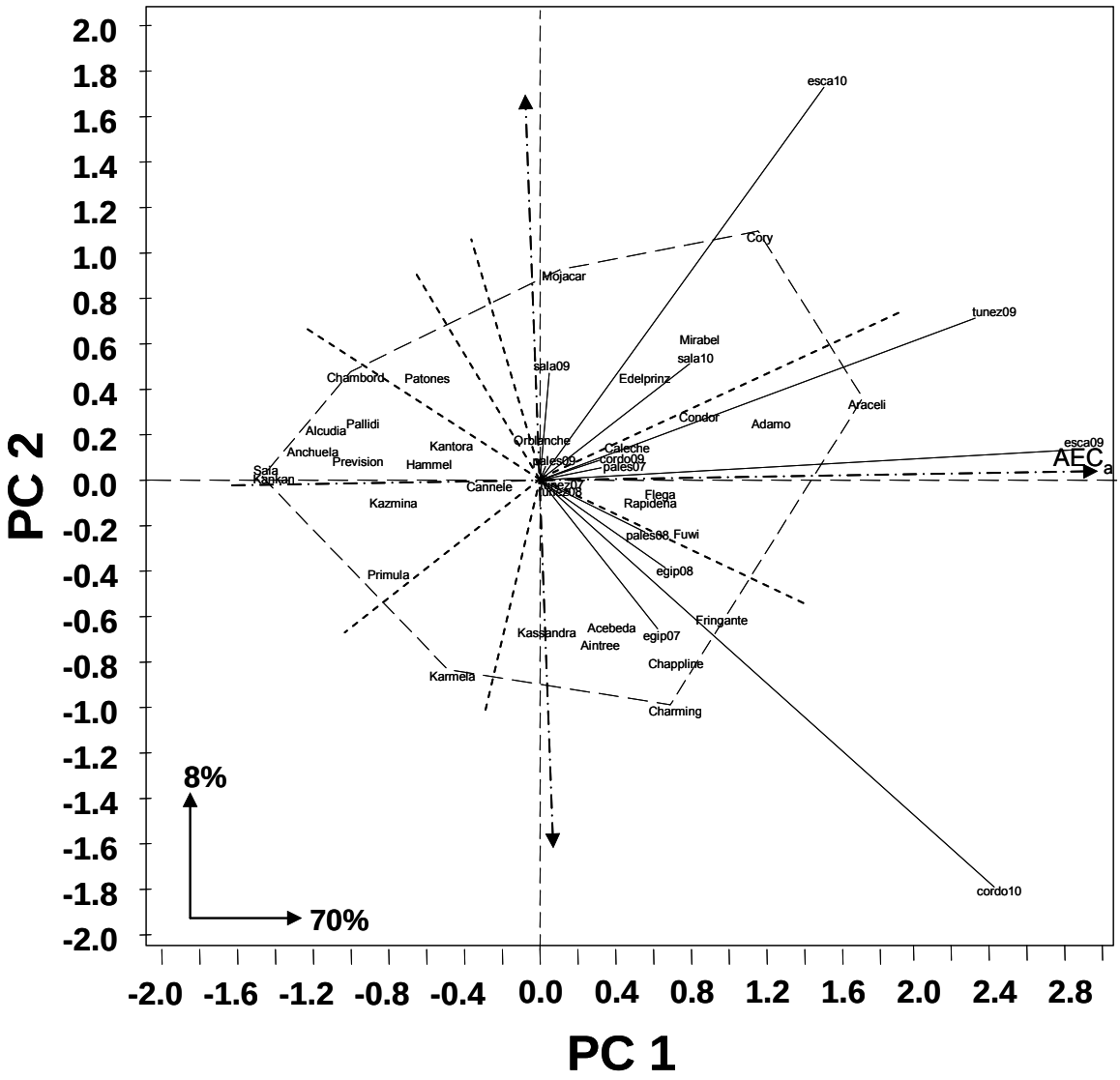
PC 2
PC 1
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0
-1.2
-1.4
-1.6
-1.8
-2.0
-2.0 -1.6 -1.2 -0.8 -0.4 0.0 0.4 0.8 1.2 1.6 2.0 2.4 2.8
8%
70%
esca10
Cory
Mojacar
tunez09
Mirabel
sala10
sala09
Edelprinz
Chambord
Patones
Araceli
Condor
Adamo
Pallidi
Alcudia
Anchuela
Kantora
Orblanche
Caleche
esca09
AEC a
Prevision
Hammel
cordo09
pales07
Sara
Kaskan
Cannele
tunez07
tunez08
Flega
Rapidena
Kazmina
pales98
Fuwi
egip08
Primula
Fringante
Kassandra
Acebeda
egip07
Aintree
Chapplipe
Karmela
Charming
cordo10

Figure 5

General Discussion

Oat is an important cereal crop cultivated over than 9 million hectares globally. However, traditionally oats have received less attention than other cereal and often have been cultivated in marginal areas with low potential productivity. Despite this, oat remains an important grain and forage crop in many parts of the world, particularly in Europe where a very significant proportion of its production is obtained in local farms. In there, many farmers and small stockholders will rely on oats in pure stand or in a mixture with peas or vetch for silage and hay, using the winter-spring soil moisture (FAO, 2011). Indeed, there is currently an increasing interest to expand the oat crop to southern regions due to its good adaptation to a wide range of soil types and because on marginal soils oats can perform better than other small-grains (Stevens *et al.*, 2004). However, oats can be sensitive to hot, dry weather and hence, in most Mediterranean and dry regions, drought is an important limiting factor to oat yield (Stevens *et al.*, 2004). In addition, crown rust (*Puccinia coronata* f. sp. *avenae*) and powdery mildew (*Blumeria graminis* f. sp. *avenae*) are major biotic constraints for oat production.

Breeding objectives vary among oat-breeding programs depending upon the particular needs of any production area. Objectives that have been typically common to most oat-breeding programs are high yield, suitable maturity, lodging resistance, good grain quality and disease resistance. However, because in northern regions rainfall does not pose a significant limitation, drought in oat has receive higher attention only relatively recently and few works on this topic have been carried out. By contrary, many more work has been carried out on oat fungal diseases, particularly to rust. However, the resistance employed has been traditionally overcome by new races of the pathogen. Particularly the gene *Pc94* that had been prove useful for the oat crown rust were recently defeated by mediterranean isolates (Jirakova and Hanzalova, 2008) highlighting the needs of new resistance sources for these highly virulent isolates. In summary, there is a need to improve oat crop according the agroclimatic conditions of the Mediterranean basin with durable and stable sources of resistance.

According to Norman Borlaug (father of the green revolution) cornerstones for breeding any plants includes: 1) as much as possible genetic sources for introduction the desired traits, 2) appropriate tools for selection of plants carrying the desired traits, 3) getting high levels of adaptation, genetic uniformity and agronomic stability (Borlaug, 1983). In this work we tackle the main constrains for the oat crop under Mediterranean environment with the aim to identify and characterise novel sources of resistance, appropriate selection tools and the information needed to ease the oat breeding programs.

To this aim, 141 oat landraces collected from across Southern Spain predominantly in the 1940s when they were used locally in agriculture and 36 currently grown commercial varieties were employed to sought and characterise new sources of resistance to both, powdery mildew and rust and drought tolerance. During the 20th century, landraces have increasingly been replaced by modern cultivars because they are high yielding under high input systems. However, this practice has narrowed the genetic base. This loss of genetic variability has been proved in a number of species; eg. maize (Warburton *et al.*, 2008) and wheat (Reif *et al.*, 2005; Roussel *et al.*, 2004). In the case of oat, its poor adaptation to water stress conditions is the result of the loss of drought tolerance traits presented in wild antecessors during its domestication process to northern regions from Fertile Crescent (Loskutov and Rines, 2011). On the contrary, landraces, despite less yielder, have considerable potential for use in improving disease and abiotic stress tolerance. This highlights the importance of screening both, landraces and high yielding cultivars.

Resistance to powdery mildew and crown rust

New sources of resistance to both fungi have been identified. In both cases the highest levels of resistance observed macroscopically were confirmed by the presence of one or more resistance mechanisms at the histological level. The histological characterization allowed identification of the specific resistance mechanisms underlying the resistance phenotype. This is of importance since: 1) they might be used as histological markers 2) it may contribute to a more durable resistance. This can be done by avoiding when possible genotypes characterised only with race-specific resistance (Prats *et al.*, 2006) and favouring the introduction of wide-spectrum resistance responses. Interestingly, some oat genotypes showed various resistance responses, hampering both fungal diseases at different stages of the infection process. In addition of using wide spectrum resistance, the use of genotypes able to stop the pathogen at different developmental stages may prove more durable and difficult to overcome by evolving pathogenic races that the use of genotypes with a single defence response (Niks and Rubiales, 2002).

Our data show that overall, commercial varieties were moderately resistant to rust but highly susceptible to powdery mildew; pre-penetration resistance was the main resistance response exhibit against crown rust whereas HR was the most common response to the powdery mildew attack. Strikingly, none of the genotypes with high resistance to rust showed also high resistance to powdery mildew. This could be expected for those genotypes showing a resistance responses based on hypersensitivity, since this kind of resistance implies a specific gen-for-gen interaction between the plan resistance gen (*R* gen) and the fungal avirulence gen (*avr*) (Mur *et al.*, 2008) and resistance responses greatly differ even between different isolates of the same fungal species. However, those genotypes displaying a broad spectrum basal resistance (i.e. penetration resistance) in the epidermal cells against powdery mildew might show a similar response against a pathogen with a similar life style such as the rust, since plant owned the necessary machinery for it. These differences between basal resistance responses to the different biotrophs demonstrate the importance of the early recognition of the potential pathogens by means of their microbial-associated molecular patterns (MAMPs) in the successful execution of the resistance. Plants possess an array of highly sensitive and specific surface receptors to monitor microbial communities according to their molecular patterns and thereby control pathogen infection (Jones and Dangl, 2006). Our results suggest a differential receptor-mediated recognition for the rust and powdery mildew derived MAMPs in the different genotypes that would allow the recognition and display of the basal defence machinery to the different biotrophic pathogens studied.

The characterization of the resistance responses reported here, may aid in the goal of achieving a more durable resistance. However, it might be tedious selecting those plants containing the desired resistance responses following histological evaluation of segregating populations. The development of genetic maps via Quantitative Trait Loci (QTL) analysis has been a major breakthrough in the characterization of quantitative traits, enabling the identification of associated genomic regions and their contribution to the phenotypic variation. Thus, identification of QTLs linked to specific resistance response might assist breeding without losing the advantage of the knowledge of the underlying resistance mechanisms. Only a

limited number of QTLs for partial resistance to crown rust have been identified and they have been determined by using the impact of the disease in agronomic traits (Diaz-Lago *et al.*, 2003) or macroscopic evaluation of disease symptoms such as disease severity and infection type (IT) (Acevedo *et al.*, 2010; Barbosa *et al.*, 2006; Jackson *et al.*, 2007; Zhu and Kaeppler, 2003; Zhu *et al.*, 2003), hence, the specific resistance responses linked to the QTLs were not elucidated. We explored the possibility to linked QTLs to specific resistance responses taking advantage of the genetic map already developed for the Ogle/TAM population (Portyanko *et al.*, 2001). Our data revealed QTLs linked to the different resistance responses, mainly for the early and late hypersensitive response (HR) but also to resistance responses developed prior mesophyll cell penetration, and therefor offer possibilities to ease breeding for oat crown rust resistance.

Despite it is widely acknowledge that the use of genetic resistance is the most appropriate way to control fungal diseases (McWilliam, 1989; Pimentel, 1982) our data shows that the execution of resistance mechanisms against *Blumeria graminis* f. sp. *avenae* and *Puccinia coronata* f.sp. *avenae* leads to physiology alterations in oats compromising its yield. Fittness costs associated with the manifestation of plant resistance to herbivores and pathogens has been studied by researcher for many years (Bergelson and Purrington, 1996) although it is attracting increasing interest only recently. Our data suggests that excess of ROS produced during the development of the resistance responses to pathogen or the inability to cope with it might be responsible of the physiological dysfunctions and hence might be a component of the resistance cost. This hypothesis will be further explored by the detailed analysis of the antioxidant machinery of these cultivars, and quantification of ROS during pathogen attack. Interestingly, the different cultivars even showing similar resistance levels did not showed the same extent of physiological alterations. This offer possibility for breeding by using oat cultivars in which the effect of resistance on plant physiology is minimised. For example in the oat powdery mildew interaction, the highly resistant cultivar Charming was the genotype with the lowest dysfunctions, whereas in the oat crown rust interaction, Kankan showed the lowest negative effects.
Overall, the histological characterization of the new sources of resistance found to both, powdery mildew and crown rust, the study on QTLs linked with specific resistance responses, and the elucidation of an important component of the resistance cost offer the material and tools necessary to ease and speed oat breeding for a durable resistance limiting yield penalties.

Drought tolerance

Following a screening of a germplasm collection, we identified 11 accessions showing moderate or high levels of drought tolerance in oat. In addition physiological characterisation of these and 3 additional moderate and susceptible accessions together with a novel multivariate approach on this physiological feature allowed the determination of specific selection criteria. This is of importance since it could be used to speed genotype selection from large breeding populations otherwise difficult and expensive to test. In fact, the lack of effective selection criteria is considered to be an important handicap to breed for drought-prone environments (Araus *et al.*, 2002; Ouk *et al.*, 2006; Venuprasad *et al.*, 2007). This is because drought tolerance is a quantitative stress with complex phenotype and genotypic control (McWilliam, 1989). In addition not all features indicative of drought tolerance are suitable for discriminating tolerant from sensitive genotypes and not all plant species respond in a similar way. For instance, water potential is not a defining feature of tolerance in faba bean (Ricciardi *et al.*, 2001) whereas it have been reported to be the main trait responsible of the drought tolerance phenotype in chickpea allowing its use as marker (Pannu *et al.*, 1993) or

in bread wheat (Schonfeld *et al.*, 1988). Thus, it remained uncertain in a given species which were the best features indicative of drought tolerance and/or when these should be assessed. Overall, this work allowed for the first time the ranking of many supposed drought resistance traits in order of degree of importance within oat, highlighting those with a causal relationship to drought stress tolerance and not only correlated with it in determinate genotypes. Thus, we identified specific water use related features such as leaf temperature and RWC as the main traits indicative of drought tolerance in oat. Other physiological processes involved in cell/tissue water maintenance including stomatal conductance and those reflecting oxidative damage and antioxidant defence albeit linked with the resistance responses might be considered as weakly correlated events not suitable for discriminating among oat genotypes. In addition, our approach was to encompass as much oat biodiversity as possible but screened under controlled conditions. Screening under natural stress conditions is difficult because of the irregular and erratic drought response (Venuprasad *et al.*, 2007) whereas controlled conditions allowed the inexpensive and robust screening of large populations with optimized protocols for selection of plants carrying specific physiological mechanisms that can be coupled later with yield assessments in the field for selected genotypes. As such, this initial stage can be readily adopted by crop breeders. By combining information on the basis of yield limitation under contrasting environments with the new physiological/ biochemical /molecular selection tools, the probability of accelerating the rate of genetic progress through plant breeding will be significantly increased (Araus *et al.*, 2002). This study shows the potential of multivariate analysis as robust approach to target key mechanisms responsible for drought tolerance in oat and can be used to speed genotype selection from large breeding populations otherwise difficult and expensive to test.

In addition of the knowledge of the physiological changes set out in oat during drought events, a deep understanding of the biochemical and molecular changes leading to drought tolerance is of fundamental importance and forms one of the major research topics. Recent work on the model plant Arabidopsis and in a lesser extent on tobacco have dissected part of the regulatory circuits leading to drought tolerance including stress sensors, transcription factors, promoters, and a large list of genes, proteins and metabolites (Bartels and Sunkar, 2005). Despite the apparent success of stress research on these model plants, the findings have been rarely applied to improved crops. One of the key reasons relates to the genetic and physiological differences between the model and crop species (Skirycz *et al.*, 2011). However, the small information available on databases for non-model crops has often limited a deeper research on these. Metabolomic studies to describe large scale metabolic changes due to a particular event may circumvent the lack of database information and then it is highly appropriate for using in commercial crops and is currently acknowledged as highly useful for dissecting key routes involved on the interaction of plant and environment (Messerli *et al.*, 2007). Therefore we used this approach to reveal novel drought-induced changes in two of the oat cultivars, Flega and Patones, previously characterized as most susceptible and tolerant to drought respectively (Chapter 6).

Our metabolomic approaches revealed a complex activation of pathway networks in Patones influencing physiological and biochemical processes leading to drought tolerance. These include 1) maintain of high relative water content by salicylate-induced regulation of stomatal movements 2) specific photoprotection mechanisms to deal with the increasing oxidative stress promoted by the low CO2 concentration consequence of the partial stomatal closure. These photoprotection mechanisms would involve both, increasing photorespiration to maintain the photosynthetic electron flux, hence, avoiding damage on PSII and increased antioxidant capacity to deal with the reactive oxygen species generated during direct oxygen

reduction in PSI. Photoprotection through oxygenation of ribulose-1,5-bisphosphate by rubisco yielding phosphoglycolate and phosphoglycerate constitutes a mayor sink for electrons (Osmond and Grace, 1995; Wingler *et al.*, 2000), in addition detoxification of hydrogen peroxide produced in the chloroplast through the oxygen reduction have been shown to be done almost exclusively by ascorbate peroxidase through the water-water cycle (Asada, 2000). Therefore, it seems crucial to couple a relatively big pool of reductants of the water-water cycle with photorespiration in order to promote an efficient photoprotection. Altogether would allow preserving membrane stability and metabolic activity in this cultivar. In Flega these pathways were not induced or not as earlier as in Patones leading to oxidative damage, reflected early as reduced PSII operating efficiency and photoinhibition, that threat the membrane stability and therefore any cellular function.

Field experiments

As previously stated by combining the new histological / physiological / biochemical / molecular selection tools, with information on the basis of yield limitation under contrasting environments the probability of accelerating the rate of genetic progress through plant breeding will be significantly increased (Araus *et al.*, 2002). For this reason we evaluated the germplasm collection over four crop seasons at 5 contrasting locations along Mediterranean Basin, including Spain, Egypt, and Palestinian Territories.

Often genotype × environment (G × E) interactions bring about discrepancies between expected and observed responses to selection due to an upward bias in the estimation of genetic variances (Haussmann *et al.*, 2001). This makes it difficult to predict the behaviour of the accessions in different situations reinforcing the need for multi-environmental testing of stability of disease resistance (Flores *et al.*, 1996). The use of GGE biplot (genotype plus genotype-by-environment interaction) removes the statistical main effect of the environment and focuses on the genotype and genotype by environment interaction components relevant to cultivar evaluation. By this approach, we have identified the most stable sources of resistance against powdery mildew and crown rust along the Mediterranean basin. In addition field experiments confirmed the potentiality of adult plant resistance to control to powdery mildew in the field along all the environments and the stability of pre-haustorial resistance against crown rust under field conditions. Regarding resistance, our results also show that appropriate screening methods under controlled conditions may offer solid information on the resistance stability and may offer an easy and cheap mean for preliminary evaluation of segregating population

Fiel data rendered Primula and Patones as the highest yielding cultivars on average over the different environments tested. Interestingly, Patones was considered as the most drought tolerant genotype under controlled conditions. The stable and good performance observed in Patones regarding yield, point out this cultivar as interesting from the breeding point of view. In summary, through physiological, phytopatological and agronomical approaches we have identified and characterised new sources of tolerance/resistance in oat to fungal diseases and drought stress that may prove highly useful for oat breeding programs for Mediterranean Basin.

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General Conclusions

1. Chapter one: Powdery mildew resistance in oat.

a. New sources of resistance to powdery mildew were found and characterised.
b. The highest levels of macroscopic resistance observed, were confirmed by the presence of one or several resistance mechanisms acting both, pre and post cell penetration, being those genotypes able to arrest the pathogen at various stages of infection particularly attractive for use in breeding programs.
c. Adult plant resistance was found in several oat accessions. Penetration resistance due to papillae defence and posthaustorial responses were the most important resistance responses contributing to adult plant resistance.

2. Chapter two: Oat resistance against crown rust virulent on Pc94.

a. New sources of resistance against a pathotype of *P. coronata* f.sp. *avenae* with virulence on *Pc*94 resistance gene were found and characterized.
b. The highest levels of macroscopic resistance observed, were confirmed by the presence of one or several resistance mechanisms acting both, pre and post cell penetration, being those genotypes able to arrest the pathogen in various stages of infection are particularly attractive for use in breeding programs.

3. Chapter three: QTL association with resistance mechanisms to crown rust.

a. Ogle and TAM 0-301, parents of the RIL studied, showed as susceptible and resistant respectively to the *P. coronate* f.sp. *avenae* used.
b. Macroscopically TAM 0-301 showed lower infection type, lower infection frequency and longer latency period than Ogle.
c. Histological studies showed resistant responses in TAM 0-301 hampering crown rust development at different stages of the infection process, both, pre and post mesophyll cell penetration.
d. Several molecular markers were found to be associated with the macroscopic traits and the specific resistant responses

4. Chapter Four: Resistance responses and plant physiology.

a. Execution of resistance mechanisms lead to physiology alterations in oat. Particularly resistance responses cause stomatal and photosynthetic dysfunctions.

b. Alteration in photosynthesis is not only due to stomatal dysfunctions but to direct damage on the photosynthetic apparatus.
c. The extent of the perturbations is not directly related to the resistance mechanism itself, i.e. hypersensitive response or penetration resistance, nor the range of resistance.
d. Execution of resistance mechanisms to powdery mildew induces higher stomatal alterations than those responses directed to hamper the crown rust, however physiological dysfunctions are comparable following challenge with both pathogens.
e. Moderate/High light intensity by itself did not affect or affect only slightly plant physiology, but when overlapped to pathogen inoculation, this increased exponentially the plant dysfunctions.
f. Alterations seems to be due to increases in oxidative stress during pathogen defense and the inability of plant to cope with it
g. The differences observed in the studied cultivars regarding physiological alterations offer possibility to breed plants to minimize the resistance cost.

5. Chapter five: Targeting sources of drought tolerance through multivariate approaches

a. New sources of tolerance to drought were identified and characterised
b. Multivariate statistical approaches indicated that two traits involved in water relations such as leaf temperature and RWC, together with AUDPC were the major source of drought tolerance being the most important discriminatory parameters.
c. This work allowed for the first time the ranking of many supposed drought tolerant traits in order of degree of importance within oat, highlighting those with a causal relationship to drought stress tolerance and not only correlated with it in determinate genotypes.

6. Chapter six: Integrated mechanism of drought tolerance in oats through metabolomic.

a. Key metabolites and pathways involved in oat drought tolerance were determined through a metabolomic approach.
b. Taken together the discriminatory metabolites suggested that biochemical pathways involved in alleviating photo-oxidative stress were a key factor for drought tolerance. Particularly those related to photoprotection and the Ascorbate-Melher pathway.

c. Metabolomic data also highlighted the importance of the main core of the phenylpropanoid biosynthesis pathway in the tolerance response. Tolerant plants subjected to drought induced high level of key metabolites of this pathway such as shikimate and their derivatives, phenylalanine, tryptophan, and salycilate (SA).
d. According to our study, increases in SA results in stomatal closure to maintain high relative water content. The potential photoinhibitory effects of this leading to the generation of ROS are reduced through increase antioxidant capacities as seen by ascorbate metabolism and increasing photorespiration to maintain the photosynthetic electron flux to avoids PSII damage.

7. Chapter seven: Multi-environment validation of resistance to crown rust, powdery mildew and drought tolerance

a. High values of total sum of squares related to the variation due to Environment (E) demonstrated that the environments were different and contrasting. All of this justifies the uses of GGE biplot method to analyze the data form multi-environment trials.
b. Although the biplots revealed genotype × environment crossover interactions, the inconsistency in environment grouping did not support the concept of megaenvironments. Therefore, the Mediterranean basin oat growing region may be considered as a single complex megaenvironment, where unpredictable crossover GE interactions are expected.
c. Rapidena and Alcudia could be considered as the highest yield producing cultivars but their performance was highly influenced by the environments.
d. Patones, Primula and Caleche were the highest biomass producing cultivars being moderately stable over environments. Patones and Primula were also the highest grain yield producing and stable cultivars, but Caleche yield poorly.
e. Saia and Kankan showed the highest and most stable resistance against crown rust, followed by Alcudia.
f. Alcudia showed the highest and most stable resistance against powdery mildew. In addition, Alcudia showed a good and stable performance for all traits evaluated.