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Developing of new stress-tolerant rice varieties for the Mediterranean region

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Memoria presentada por Eduardo Ramos de Fuentes para optar al grado de doctor por la Universitat de Barcelona. Este trabajo se enmarca dentro del programa de doctorado "Ecología, Ciencias Ambientales y Fisiología Vegetal" correspondiente al bienio 2015/2016 del Departamento de Biología Evolutiva, Ecología y Ciencias Ambientales, sección de Fisiología Vegetal, de la Facultad de Biología de la Universitat de Barcelona.

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Doctorando	Director de tesis
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CÀMARA ARROCERA DEL MONTSIÀ — MONTSIÀ

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Imagine there's no countries…

Nothing to kill or die for…

Imagine all the people living in peace

John Lennon - Imagine

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ABBREVIATIONS

BC	Backcross
FAO	Food and Agriculture Organization
IRRI	International Rice Research Institute
KASP	Competitive allelic-specific PCR
LP	PL12 x FL478 cross
LS	PL12 x IR64-Saltol cross
MABC	Maker Assisted Backcrossing
Mb	Megabases
MP	PM37 x FL478 cross
MS	PM37 x IR64-Saltol cross
PCR	Polymerase chain reaction
QTL/QTLs	Quantitative trait Locus/Loci
RCP	Representative Concentration Pathways
SES	Standard Evaluation System
SME	Small and Medium Enterprises
UNF	Unit of nitrogen fertilization

SUMMARY

Rice (*Oryza sativa*) is the most important crop for human direct consumption, but its yield and production are strongly affected by biotic and abiotic stresses. Rice is the most salt-sensitive cereal and in addition, salinity is a rising problem around the world reinforced by the climate change effects such as rise of the sea level, soil degradation and water scarcity. Regarding biotic stresses, the apple snail (*Pomacea sp.*) is one of the worst introduced pest that affects rice production. It has been detected for first time in Europe in Spain, in 2009 in Ebro River Delta, destroying rice fields at seedling stage. Additionally, rice blast, caused by an ascomycete called *Pyricularia oryzae*, is a disease that strongly affects the rice production worldwide causing yield losses that range from 15% to 50%, even using specific fungicides.

The main objective of this thesis is to develop new stress-tolerant rice varieties for the Mediterranean region, by the introgression of the *Saltol* QTL and new blast resistance genes to Mediterranean local varieties.

A molecular marker assisted backcross scheme (using KASP technology) was followed to introgress the salt tolerance traits. The *Saltol* donor varieties were FL478 and IR64-Saltol, two salt-tolerant Asiatic *indica* rice lines, while the recurrent parental lines were PL12 and PM37, two Mediterranean japonica rice lines. Manual forced hybridization between the donor and recurrent lines was performed, being assisted by embryo rescue on immature seeds to speed up the process. A foreground genetic selection was performed for each generation, with a single SSR marker. Background analyses and selection, meaning the KASP molecular marker analyses, were performed in BC_2F_1, BC_3F_1 and BC_3F_2 generations in order to select those individuals presenting the highest return to the recurrent parent genome.

BC_3F_3 plants were tested for salt tolerance in hydroponic assays, adapting the design and solution from Yoshida and International Rice Research Institute (IRRI) works. The standard evaluation system (SES) described by IRRI was used to evaluate the lines.

Relative chlorophyll content (RCC, measured with a SPAD), fresh weight and plant length was also recorded. Additionally, two consecutive years of field assays were performed in Ebro River Delta using different foreign and local lines (and their hybrids), to evaluate their general performance and rice blast resistance.

From the 4 crosses combination performed between both *Saltol* donors and both Mediterranean recurrent parents, PL12 x FL478 (LP cross) and PM37 x IR64-Saltol (MS) were selected to proceed with the whole backcrossing process, to determine the return to the recurrent parent genome and to obtain the homozygous *Saltol* BC_3F_4 seeds. More than one thousand plants were generated for each cross, but at the end, 18 BC_3LPF_3 plants and 10 BC_3MSF_3 homozygous *Saltol* plants which had reached between 95% and 98% of return to recurrent parent genome were selected.

For the hydroponic assays, 54 BC_3LPF_3 lines were initially tested since a high variability between lines was observed. From them, some lines like LP-3, LP-15 or LP-17, performed similar or even better in the SES than the salinity donor FL478 line.

The RCC data showed again a high variability between lines, although RCC did not correlate with SES results. The data analysis was hindered due to the differences scored between replicates and the fact that much of them were totally dead at the end of the assay. A certain degree of heterozygosity may explain the variability found between replicates during the SES evaluation.

The fresh weight (FW) and the plant length in both shoot and root was strongly affected by the salinity treatment. However, the reduction was higher in shoot, where some lines account a 90% of weight reduction and a 60% of length reduction.

Finally, the blast tolerance field assays were severely affected by the rice stem borer *Chilo suppressalis*, both years. This pest is not a big problem in Ebro River Delta, where local lines are adapted, but foreign cultivars are especially susceptible. The evaluation for rice blast tolerance was really difficult and no outstanding line was selected to proceed.

In conclusion, the *Saltol* QTL has been successfully introgressed in two Mediterranean japonica rice varieties, although more replicates of the hydroponics assays must be performed to confirm and select the most salt tolerant obtained lines. These lines will be tested in 2018 and 2019 in field assays, under salinized and no salinized conditions. Regarding rice blast resistance field assays, no conclusive results were achieved. More field assays must be done, and other lines should be tested. In order to avoid or reduce *C. suppressalis* infestations, more phytosanitary actions should be taken in the future.

INTRODUCTION

1. INTRODUCTION

1.1. Rice, a food crop

i. Rice production, consumption and relevance worldwide

Rice is the fourth crop in terms of world production, behind sugar cane, maize and wheat [1]. Paddy rice reached an all-time high production of 751.9 million tonnes in 2016 (501 million tonnes milled rice). It represents a 1.8% increase from 2015 production and the forecast for 2017 expects to reach the 754.6 million tonnes [1].

Unlike sugar cane and maize, which are mainly used for biofuel production and animal feed respectively, rice production is mainly dedicated to human direct consumption (*ca*. 75%) (Figure 1) [2]. Rice is the most important source of calories for humans, contributing approximately 21% of world per capita caloric intake, but reaching 80% per capita caloric intake in high consumption countries like Vietnam, Cambodia or Myanmar [3]. It feeds almost 50% of the world population each day, and it is the staple food in the poor areas of Asia, Africa and South America. Thus, rice is probably the most important crop for human population [3,4].

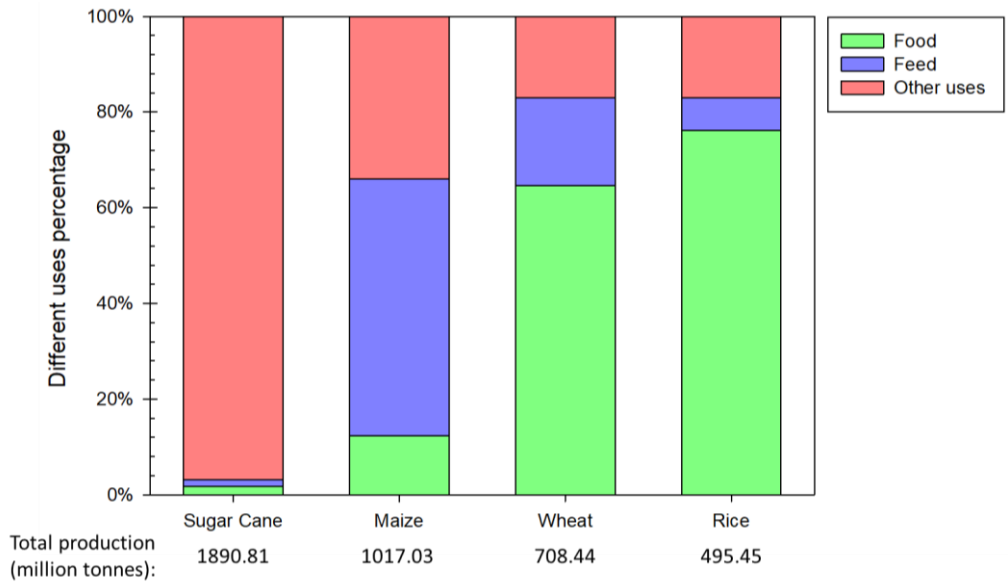

Figure 1. The World's main crops total milled grain productions in 2017 and their utilization in food (green), feed (blue) and other uses (red). Data from FAOSTAT [2]

Excepting the Antarctica, rice is cultivated in all continents, being Asia the main producer (90.49% of global production), specifically in tropical and subtropical regions such as south China (*ca.* 200 million tonnes), India (*ca.* 150 million tonnes), Indonesia (*ca.* 100 million tonnes) or Bangladesh (*ca.* 50 million tonnes) [4,5]. The remaining <10% of rice, is cultivated in temperate regions such as Australia, United States or Europe, usually under Mediterranean climate conditions.

Europe achieves less than 1% of the global production. Nevertheless, its quality aspects make it an important product for the region. European rice production is mainly consumed by Europeans (no more than 20% is exported) and represents the 61% of total rice consumed in Europe [2,6,7]. Italy and Spain are by far the biggest European producers, with *ca.* 50% and 30% of total production respectively. Other European rice producers are Portugal, France and Greece (Figure 2).

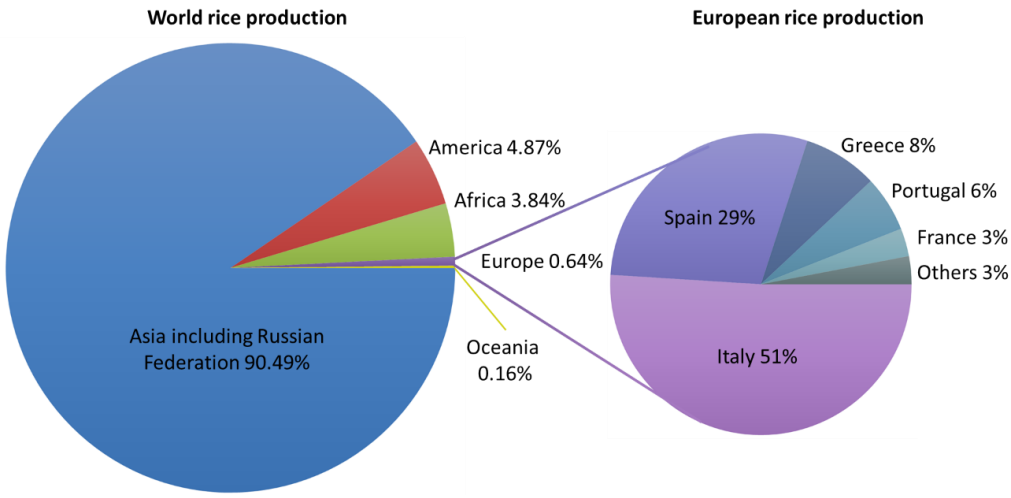

Figure 2. World rice production by continent and European rice production by country [2,8].

In the international market, the long grain rice is the most common, but in spite of that, long grain production in Europe represents only the 25%. The short-medium grain (*japonica*) represents the 75% of the European production since the European rice

market has switched towards more local production of short-medium-grain rice in the recent years, being the most of the long grain rice (*indica*) imported [5,9,10].

Thus, rice is a crop that has influenced Europe in many aspects, with obvious economic and ecological impacts (e.g. the recovery of Deltas as marshlands), but also in the society and culture. For example, some countries have adapted their gastronomy to locally produced varieties, in general of short and pearl-shaped grain (japonica rice), which are the best suited for *paella* or *risotto* recipes. This specifically short and pearl-shaped grain is principally grown in Europe, because the pearl is seen as an imperfection in the rest of the world, sometimes mistaken with chalky rice [11].

In Spain, the rice production is distributed in three major regions: Guadalquivir-Guadiana Rivers in South Spain (60% of total production), Ebro Delta in Catalonia (16%) and Albufera in Valencia (14%) [12]. Most of the rice production in Spain is based in cooperative associations, which grants the production to the farmers, boost the local economy and influences the industrial and business networks. These cooperatives usually not only produce milled rice, but also certified seed and invest in new varieties development and other research projects [12] .

For the last 50 years the rice yield and its harvested area has raised steadily [5,9]. Nevertheless, the global population growth rate has not slowed down, which poses a future problem in global food security. Moreover, biotic and abiotic stresses force farmers to use more inputs (pesticides, herbicides and fertilizers) every year, which reduces profitability and increases the rice price [4]. Thus, it is necessary to further investigate and develop enhanced rice varieties in terms of production and stress-tolerance.

ii. *Oryza sativa*, origin and cultivation

Rice is a member of the *Poaceae* family that belongs to the genus *Oryza*. There are more than 20 *Oryza* species, although only two are cultivated: *Oryza sativa* (cultivated around the world) and *Oryza glaberrima* (cultivated residually in West Africa) [13]. *Oryza sativa* has two subspecies, *O. sativa ssp. indica* and *O. sativa ssp. japonica.* These subspecies are further classified in five genetic groups, *indica* and *aus* under *O. sativa ssp. indica* and temperate, tropical and aromatic under *O. sativa ssp. japonica* (Figure 3) [14].

Popular tales and mythology attribute domestication of rice to the legendary Chinese emperor Shennong, the God of Agriculture, 4.500 years ago [15]. The real origin and domestication have been controversial and long debated [16–21]. Recent genetic evidences suggest that rice was domesticated from *O. rufipogon* near the Pearl River, southern China, 8200-13500 years ago. Firstly, the subspecies *O. sativa japonica* was obtained and then *O. sativa indica* was developed from crosses with local wild rice from South East Asia [17,18].

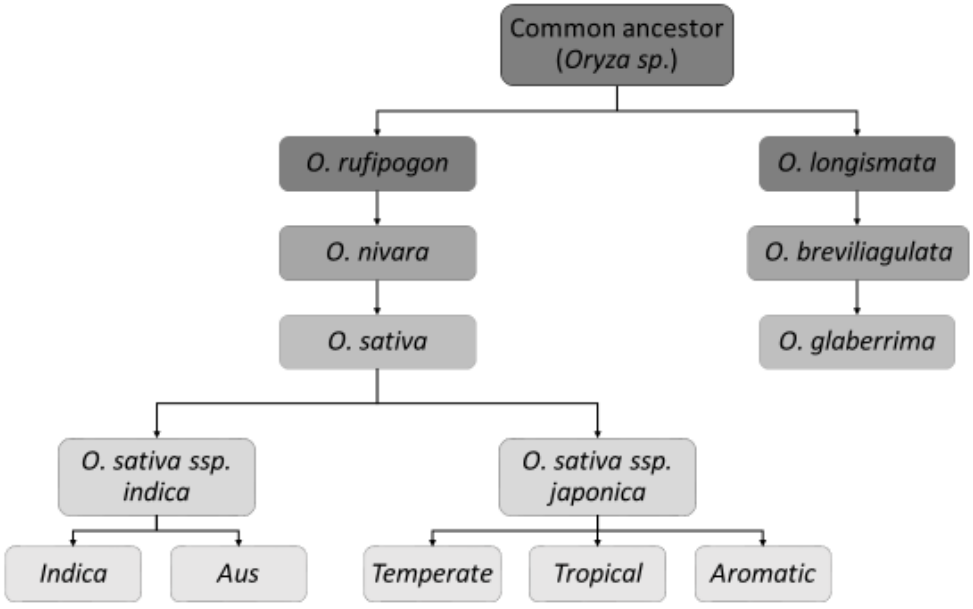

Figure 3. Genetic groups of *O. Sativa*. Adapted from Garris et al [14].

The major development and evolution of rice were performed on China and South East Asia, not only in the seed (plant and crop characteristics) but also in the management. In the Neolithic, rice was directly seeded in forest clearings, without standing water. With the agricultural development and the first settlements near rivers in China, puddling the soil (turning into mud) and the seedling transplanting were refined. These techniques increased the yields and contributed to human population growth [4].

From China and South-East Asia, rice cultivation was diffused to Indonesia about 1500 B.C. and to west India and Sri Lanka about 1000 B.C. From there, it is supposed that the crop was introduced in Greece and the Mediterranean by returning members of Alexander the Great's expedition to India around 330 B.C. Rice was later spread all over the Mediterranean by the Muslim Empire. Rice also arrived to Japan no later 100 B.C. Years later, with the Age of Exploration, rice cultivation was introduced in the New World by European settlers [4,22].

Nowadays, rice is cultivated in a wide range of locations under a broad variety of climatic conditions, from the wettest areas, like Myanmar's Arakan Coast (where more than 5100 mm of rainfall are recorded per season) to the driest deserts, like Al Hasa Oasis in Saudi Arabia (where annual rainfall is lower than 100 mm). Temperature is also a factor that varies greatly, from an average during growing season of 33ºC in Pakistan, to 17ºC in Japan. Furthermore, the crop is usually cultivated at sea level, in coastal zones, delta regions and river basins, but also cultivated at a height of 2600m on Nepal's mountains [4,22].

As commented before, Asia is the world greatest rice producer, but mostly because it concentrates more than 80% of harvested area. In fact, the highest yields are obtained in high/low-latitude areas that have long day length, high solar irradiation and cold nights. Nile River Delta in Egypt, South-western Australia, Northern California in United States and Spain are the best examples (Figure 4). This fact is also in part explained by the different field managements used around the world. In South Asia the crop is produced in small plots using plenty of human labour, while in other locations such as

United States and Australia, rice is cultivated in large fields and raised with top technologies and huge consumption of fossil fuel energy [2,4].

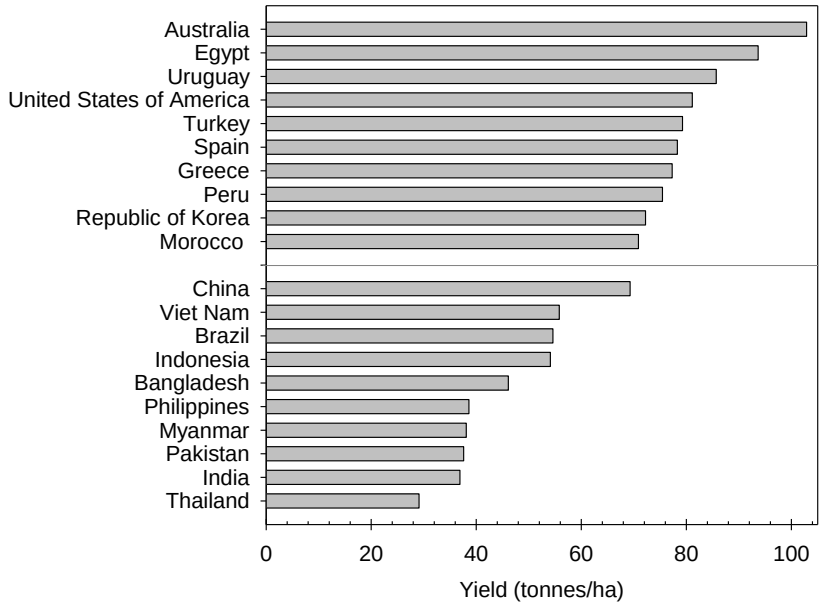

Figure 4. Graphic showing the yield from the ten countries with world highest yield (Australia to Morocco) and the ten countries with world highest total production (China to Thailand), in tonnes/ha [23].

In the recent decades, extreme, erratic and adverse weather is affecting rice cultivation [1]. Periods of severe drought followed by heavy rainfall and tropical storms have provoked floods and destruction of fields in some areas of South America and Asia, usually below the Equator. Moreover, rise of the sea level is threatening large cultivation areas, and causing the increment of the salinized phreatic layer in the coastal fields. In general, climate change is a factor to consider, and to be followed closely, in the future of rice cultivation [1].

1.2. Climate change and its consequences

Last reports from the Intergovernmental Panel on Climate Change (IPCC) show some future scenarios depending on how much annual greenhouse gases (GHG) emissions are reduced. In the best case, the Earth temperature will increase between 1-2°C to 2100, following the Representative Concentration Pathway 2.6 (RCP 2.6), which implies negative anthropogenic CO_2 emissions after the year 2070. Maintaining the actual anthropogenic CO_2 emissions will assure more than 2°C of global temperature increase [24] (Figure 5).

The industrial revolution caused a greenhouse gas emissions increment, being mostly CO_2 resulting from the fossil fuels combustion. This phenomenon has altered the energetic equilibrium of the atmosphere, in which these gases generate a greenhouse effect that maintains the planet warm and allows the life in it, but their over-accumulation has caused a global warming [24].

Since the preindustrial era (1850s) to the present day, the mean global temperature has increased in 1°C, which is concerning since the complete climatic system of the Earth depends on the temperature. The rise of temperature in the oceans is affecting the wind flows and clouds, which in turn causes an increase of extreme and abnormal weather. Moreover, global warming has caused a severe reduction in the Artic sea ice reserves, which has induced the rise of the ocean level (mean rate of global averaged sea level of 3.2 [2.8 to 3.6] mm/year between 1993 and 2010), as well as a change in its pH and dissolved salts [24].

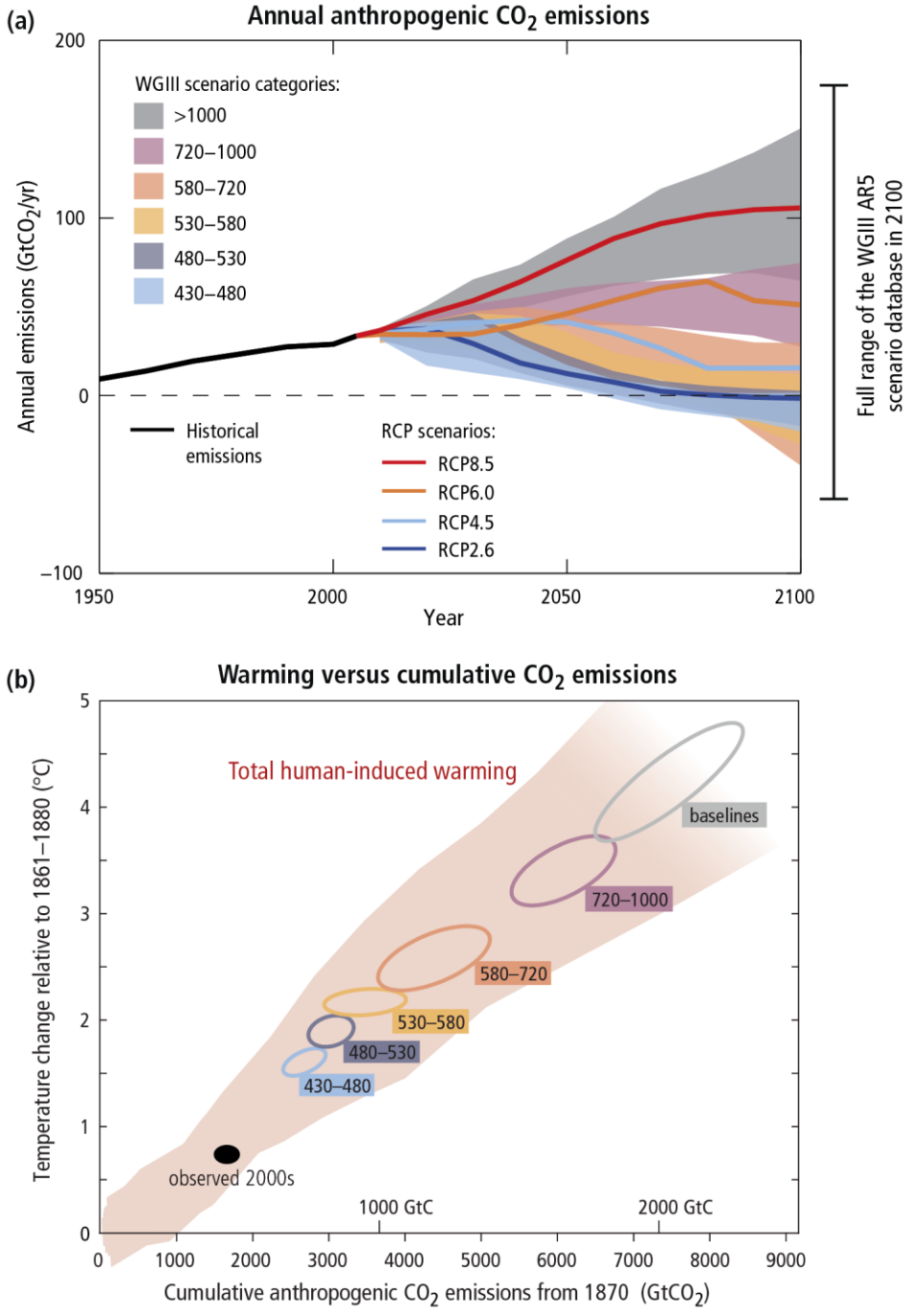

Figure 5. a) Emissions of carbon dioxide (CO_2) alone in the Representative Concentration Pathways (RCPs) (lines) and the associated scenario categories used in WGIII (coloured areas show 5 to 95% range). b) Global mean surface temperature increases at the time global CO_2 emissions reach a given net cumulative total, plotted as a function of that total, from various lines of evidence [24].

There is one clear and critical consequence of climate change: the forced relocation of communities. In 2014, the village of Vunidogoloa, in Fiji, was relocated 2 km inland after years of inundations because of the rise of sea level [25]. This is the first case of climate change refugees but some other villages and towns are addressing the same problems to the authorities, like Kivalina town in Alaska (USA), the Guna people of Panama in the Caribbean coast, or the Quinault Indian nation in Washington (USA) [26,27].

The United Nations is already aware that climate change will generate millions of refugees, not only because of the direct loss of land, but also because of the remaining land degradation [28,29]. The world's cultivable land is being degraded, mostly via salinization, due to the consequences mentioned above, combined with some anthropogenic factors [30,31]. This soil degradation threatens the food security and could lead to the starvation of communities and the migration of millions of people.

1.3. Salinity, a major threat for crops

i. Causes of soil salinity and distribution of affected soil

Climatic change is promoting the rapid soil degradation in agricultural lands worldwide. Soil salinization highly contributes to this phenomenon and can arise from natural causes like sea level increase or land subsidence, but also from human-mediated activities such as irrigation in arid or semi-arid regions or fertilization excess [32].

About 20% of the world irrigated lands are affected by soil salinization, up from 15% in the early 1990s [30,33,34]. Salinity stress significantly reduces growth, productivity and/or quality of the majority of crops. Yield reductions range from 20% to 50% in average, being some species more affected than others by this effect. In particular for rice, salinity interferes with its growth and development, plant adaptation and stress responses. For example, salinity affects rice cultivation in key aspects such as seed germination, seedling establishment, number of tillers per plant, percentage of sterile spikes and grain maturation [33,35–38].

Salinity is a term that represents all the problems of a soil accumulating excessive amounts of salts. Sal-affected soils are usually classified as saline, saline-sodic or sodic soils using Electrical conductivity (EC) and exchangeable sodium percentage (ESP) as seen in Figure 6. Soils that are not greatly salt-affected are classed as normal. The processes that result in the accumulation of neutral soluble salts are referred to as salinization. The salts are mainly chlorides and sulphates of calcium, magnesium, potassium and sodium. [32,39]

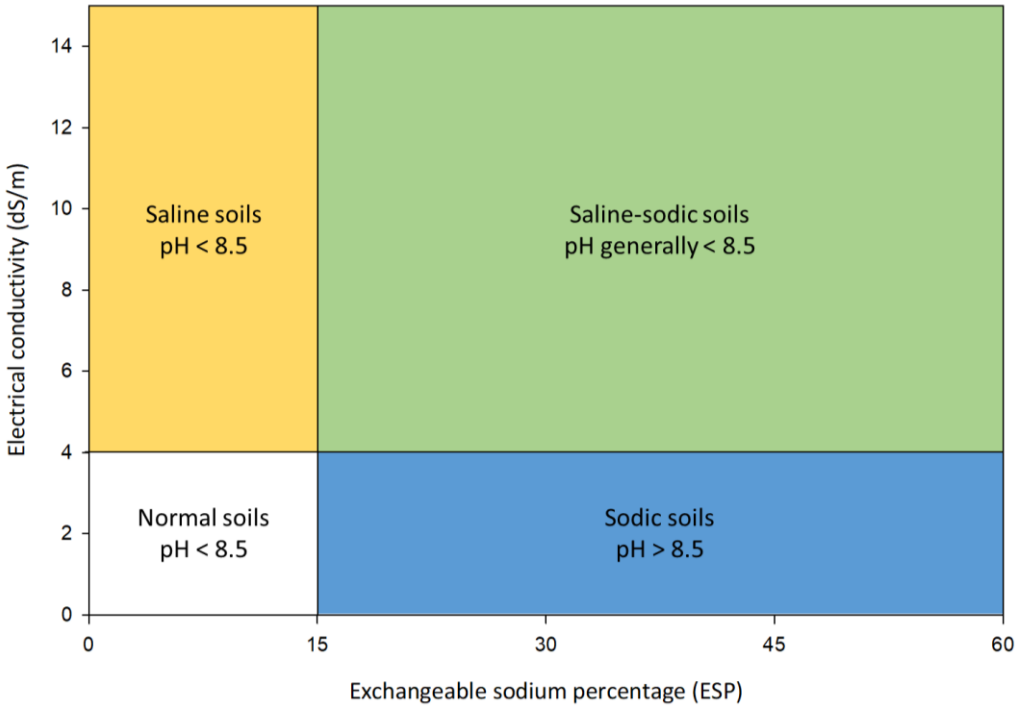

Figure 6. Diagram illustrating the classification of normal, saline, saline-sodic and sodic soils in relation to soil pH, electrical conductivity and exchangeable sodium percentage (ESP). Adapted from [39].

Saline soils are those soils that contain sufficient salinity to give EC values greater than 4dS/m but have and ESP less than 15. Thus, the exchange complex of saline soils is dominated by calcium and magnesium, not sodium. The pH of saline soils is usually below 8.5. Soluble salts helps in preventing the dispersion of soil colloids, so plant

growth on saline soils is not generally constrained by poor infiltration, aggregate stability or aeration [39].

Soils that have both detrimental levels of neutral salts (EC greater than 4dS/m) and high proportion of sodium ions (ESP greater than 15) are classified as saline-sodic soils. Plant growth in theses soils can be adversely affected by both excess salts and excess sodium levels. The high concentration of neutral salts moderates the dispersing influence of the sodium [39].

Sodic soils (or alkaline soils) are, perhaps, the most troublesome of the salt-affected soils. While their levels of neutral soluble salts are low (EC less than 4.0 dS/m) they have relatively high levels of sodium on the exchange complex (ESP are above 15). The pH values of sodic soils exceed 8.5. These extreme pH levels are due to the fact that sodium carbonate is much more soluble than calcium or magnesium carbonate. The extremely high pH levels may cause the soil organic matter to disperse and/or dissolve. The main reason for the poor plant growth is that few plants can tolerate the extremely poor soil physical conditions and slow permeability to water and air in sodic soils [39].

Rice that is grown on coastal areas is usually grown in saline-sodic soils, where an EC of 4 dS/m is considered as moderate salinity and causes a heavy growth reduction to the crop. More than 8 dS/m become high salinity that could kill the plant if maintained for more than one week. In some areas rice can be cultivated in alkaline soils, where a pH 9.3 - 9.7 is considered a moderate stress and above 9.7 strong stress. [32,33].

Taking the Ebro River Delta as a proximity example, soil analyses from different salinized fields showed a soil EC above 6 dS/m at a depth of 5cm and an ESP above 17 (IRTA personal communication). The future projections regarding soil salinity are not really favourable to rice production in this region. The models predict a rise of 1 – 8 dS/m mean soil salinity, depending on distance from river and delta sea shores, clay presence and surface elevation (Figure 7). Thus, it is predicted a maximum reduction in normalized rice production index from 61.2% in 2010 to 33.8% by 2100 in the worst scenario (RCP8.5) [40].

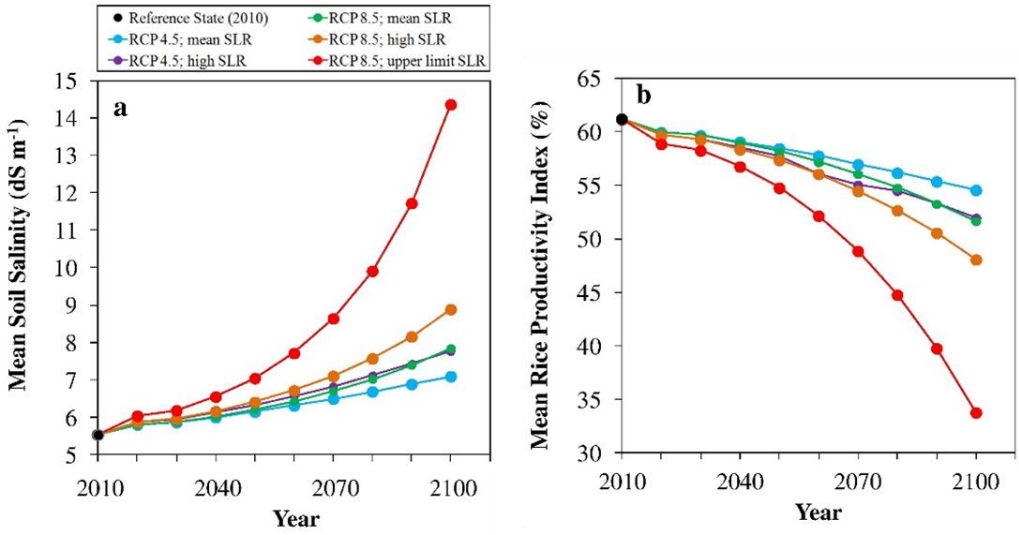

Figure 7. Estimated a) Mean soil Salinity (dS·m-1) and b) Mean Rice Productivity Index (%) along the 21st century under different simulated scenarios of sea level rise (SLR). Adapted from [40].

ii. Saline stress in rice and salinity tolerance mechanisms

In plants, salinity causes two major stresses, first an osmotic stress and later an ionic stress (Figure 8). The osmotic stress affects plants yet when the salt levels rise above a threshold outside the roots, causing stomatal closure and inhibition of water uptake, cell expansion, and bud development [33,35,41]. This threshold depends on the specie and in the case of rice, the most salt-sensitive cereal, it is below 40 mM NaCl [33]. The consequences for a rice plant exposed to osmotic stress is a reduction of growth, which is more severe in shoot than in root. An explanation could be that the reduction in the leaf area development relative to the root growth would decrease the plant water use, thus allowing to conserve soil moisture and preventing salt concentration escalation in the soil [35,41].

The ionic stress starts when the salt accumulation reaches toxic levels in the older leaves and triggers premature senescence. When the leaf senescence rate exceeds the new leaves production, the plant will not be able to maintain the carbohydrate needs and will collapse. Moreover, the ion misbalance inhibits essential functions such as

photosynthesis, protein synthesis and enzymatic activity, affecting the whole plant growth. In terms of growth reduction, the ionic stress is commonly less severe than the osmotic stress imposed by salinity, although at extreme salinity levels or in some salt-sensitive species the ionic stress could dominate the osmotic stress effects [33,35,41].

Thus, there are two main targets in salt tolerance, the osmotic adjustment and the ion homeostasis. In rice, genotypic differences have been linked between the osmotic adjustment and the growth under stress [37,41,42]. This osmotic adjustment can be achieved by accumulating ions, solutes and/or organic compounds in the vacuoles and by regulating the water uptake with aquaporins. The regulation of that tolerance is complex and comprehends diverse QTLs and genes [41,43,44]. Regarding the ion homeostasis, it is based in the extrusion, compartmentation and reabsorption of Na^+ ions. The ion transporters diversification in cell membranes is an adaptation to avoid the Na^+ ions penetration in the root cells, xylem and vacuoles. This tolerance mechanism is usually regulated by known genes and loci that codify for Na^+/K^+ -ATPases, H^+ -pump ATPases, H^+/Na^+ antiport and high-affinity K^+ uptake transporters among others in diverse membranes within the cell (plasmatic, vacuolar and nuclear), but also by calcineurin B-like proteins (CBL) and CBL-interacting protein kinases. Some of these loci are the salt overly sensitive (SOS), NHX1-like gene and the HKT-like family of genes, which in rice includes OsHKT1;5 (also known as SKC1), an important gene that codifies for an Na^+ transporter that is predicted to reabsorb Na^+ from the xylem [33,35,41,45].

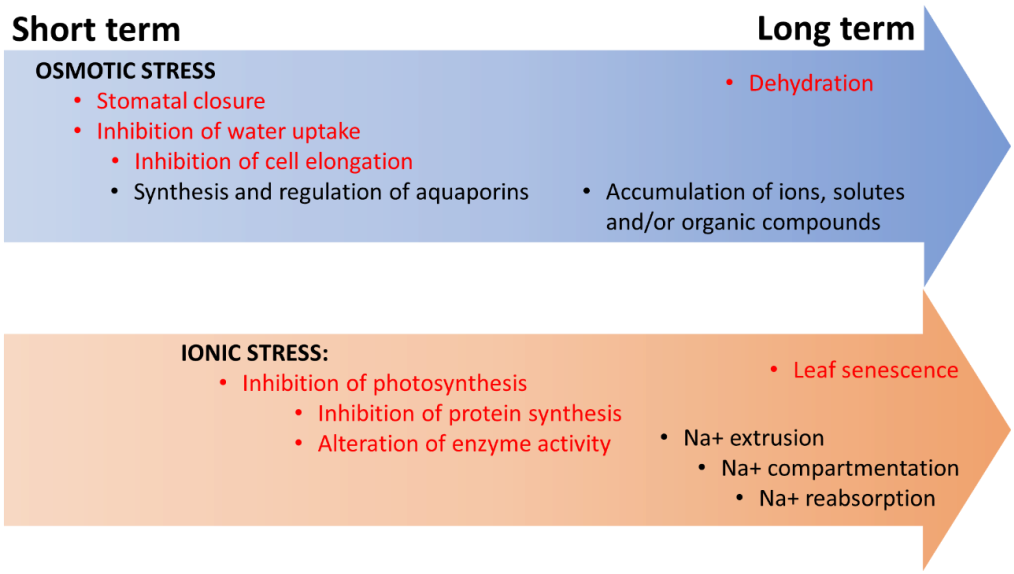

Figure 8. Schematic representation of the consequences of salinity stress in glycophytes (red labels) and some responses (black labels), at short and long term. Adapted from [46].

iii. *Saltol*, a QTL of salinity tolerance

In the last decade, salt tolerance in cereals has been a subject of study by many research groups and companies, which ultimately wanted to apply their knowledge on salinity stress and tolerance mechanisms to the agricultural reality [47–49]. Following this objective, some groups such as the ones in the IRRI have not only discovered new salt tolerance QTLs but they also have introgressed them into elite varieties [50].

In 2005, an *Oryza sativa* F_2 population obtained from the cross between a salt-tolerant landrace named Nona-Bokra and an susceptible elite variety, Koshihikari allowed to identify some rice salt tolerance QTLs [51]. Among them, one stood out, the SKC1 QTL (later named as *Saltol*). Another study displayed a RILs population between Pokkali, a salt-tolerant landrace and IR29, a salt-sensitive elite variety, which confirmed the position and involvement of the *Saltol* QTL in the salinity tolerance. This QTL explained 43% of the variation for seedling shoot Na-K ratio in this population [52–54]. These studies have allocated this QTL in the short arm of the chromosome 1, which contributes to salt tolerance via ion homeostasis (maintaining a healthy Na^+/K^+

equilibrium). However, the RILs obtained from the Pokkali/IR29 cross had a high diversity in *Saltol* QTL alleles, making it difficult to identify the source of the tolerance [53,55]. In-depth studies identified a pectinesterase and two candidates genes inside the *Saltol* QTL: SKC1 (or OsHKT1;5, a sodium transporter) and *SalT* (a salt stress induced protein of unknown function) (Figure 9) [56].

The HKTs proteins belong to a family of ion transporters which are well described in other species such as *Arabidopsis thaliana*. The OsHKT1;5 gene is similar to the *A. thaliana* gene AtHKT1.1 and the wheat TaHKT1.5 gene, which codifies to Na^+ and Na^+/K^+ membrane transporters respectively. The research done in these transporters suggest a Na^+ reabsorption function at xylem parenchyma cells, maintaining a high K^+/Na^+ ratio in leaves, which results in salt tolerance of the plants during salinity stress at seedling stage [33,41].

The introgression of the *Saltol* QTL in elite rice varieties has resulted in a promising strategy to increase salt tolerance in any rice cultivar. Among the RILs generated from the IR29xPokkali cross, the FL478 RIL featured the highest salt tolerance (>100 mM) [56]. However, Pokkali introgressions within FL478 conferred some undesirable traits such as red pericarp, dehiscence, and high height. This line was later introduced as salt tolerant donor in a backcross program to introgress *Saltol* in IR64 elite cultivar from Philippines. The resulting IR64-Saltol line showed a moderate salt tolerance (>80 mM) but lacking those undesirable traits present in FL478 [50]. FL478 has also been used to increase salt tolerance of Pusa Basmati 1121 in the same manner [57], and is used now in Europe to generate new European salt tolerant rice varieties in the H2020 project NEURICE [58].

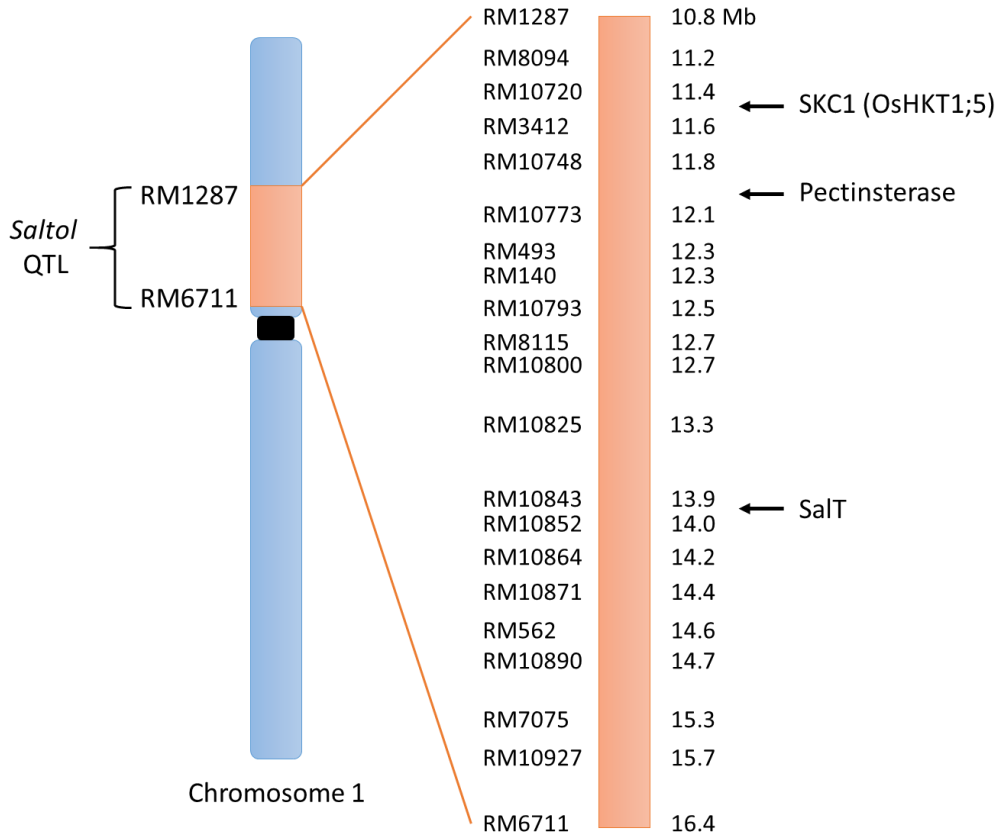

Figure 9. *Saltol* QTL region on the short arm of chromosome 1. Twenty-one polymorphic SSR markers are shown with the physical map position in megabases. Three candidate gene loci were targeted for developing gene-based markers although only SKC1 was confirmed to be a salt tolerance gene [59].

1.4. Apple snail, a new pest in Europe

i. *Pomacea sp.* biology and significance around the world

Apple snail is the common name used to represent the *Ampullariidae* family of freshwater snails. This family includes some species from the genera *Pomacea*, *Pila* or *Marisa* that have become invasive species. The Apple snails can eat any vegetation in lakes and wetlands, causing severe environmental and economic losses. In rice paddy fields they can also provoke devastating losses as they eat rice seedlings. They represent one of the worst mollusc pests worldwide, and there has been limited success in their control in rice paddies with hand picking and pesticides [60].

Ampullariidae snails are predominantly distributed in humid tropical and subtropical regions of Africa, South and Central America and Asia. It comprises nine genera with almost 200 species, although some of them seem to be synonyms and could be reduced with more research [60,61]. This family includes the largest of all freshwater snails (up to 17 cm in maximum dimension) and are a major component of the native freshwater mollusc faunas of many regions. Their life span varies among species, being three years the usual longevity of *Pomacea*, *Pila* and *Marisa* species [61].

Apple snails are extremely well adapted to tropical regions having drought periods alternated with rainfall periods. This adaptation is reflected in their habits, being moderately amphibious (although it generally has limited movement out of water) and being equipped with an operculum, enabling the snail to close its shell to prevent drying out while hiding in the mud. Another characteristic of the apple snails is the combination of branchial respiration systems (comparable to the grills of a fish) and a lung, for aerial respiration, which expands the action radius of the snail in search of food [60].

Even that many snail species are hermaphrodite, apple snails are not, they have separated sexes and a male and a female are needed for reproduction. Reproduction in *Ampullariids* may occur if water is available and temperatures are high during summer in temperate regions or throughout the year in many tropical and subtropical regions, allowing continuous reproduction in such latitudes. Each egg clutch can have from 100 to 1000 eggs depending on the species, and are usually bright pink, green or white coloured (Figure 10) In general, apple snail species lay the eggs above the waterline, in a calcareous clutch on plant stems, human structures or any consistent floating things, such trunks or tree branches, to protect their eggs against predation by fish and other water inhabitants. Some apple snail genera such as *Pomacea* and *Pila*, have a tubular siphon to breathe air while staying submerged, thus avoiding bird predators in low oxygen content water [60].

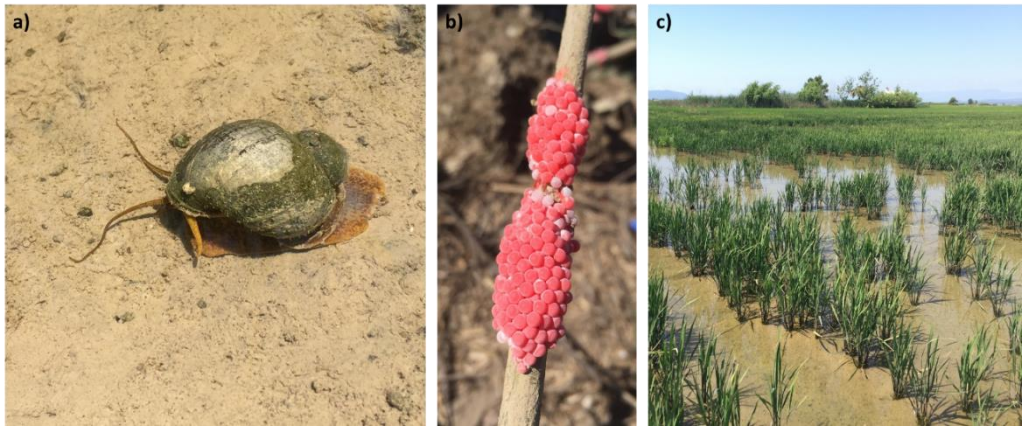

Figure 10. Original images taken in Ebro River Delta, Spain, in summer 2015. a) Apple snail (*Pomacea sp.*) individual b) Apple snail eggs and c) Damages caused by an infestation of apple snail in a field near *Deltebre*.

However, in their native areas, the apple snails have many and diverse predators, from insects to birds, including fishes, amphibians, reptiles, turtles, and mammals. The majority of them are casual feeders, but some feeds almost exclusively of apple snails, like the caiman lizard (*Dracaena guianensis*), the everglade kite (*Rostrhamus sociabilis*) or the limpkin (*Aramus guarauna*) [62].

Only species from the genus *Pomace*, *Pila* and *Marisa* have become invasive with terrible economic consequences. Their invasiveness is easily explained because they exhibit high reproductive potential, fast growth rate, high dietary flexibility and strong resistance to a number of environmental conditions (including hypoxia, high temperature and desiccation) [63–65]. *Pomacea* is the genus that more non-native ranges occupies, mostly because of the introduction of *P. canaliculata* and *P. maculata* in South-East Asia and Europe as food and aquarium pet (Figure 11) [60,66,67].

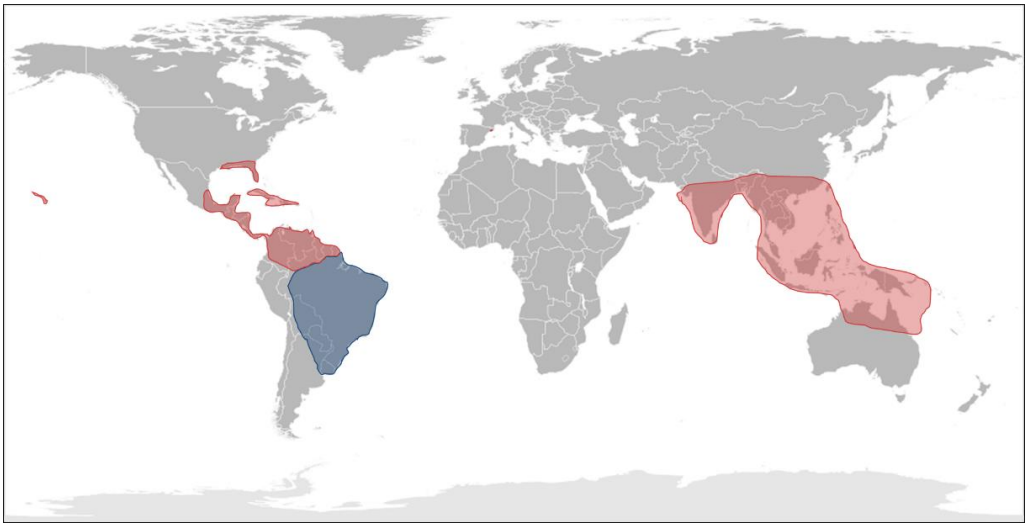

Figure 11. Native (blue) and non-native (red) distribution of *Pomacea* genera. Along with Centre-America, South-east Asia and Hawaii, Spain has been invaded by *Pomacea* species. Adapted from Hayes et al. [61]

ii. Damage caused in Spain and eradication actions.

The apple snail was first detected in Europe in 2009, near the locality of *l'Aldea* in Ebro Delta, Spain. It expanded infesting all the north hemi-delta via canalizations and drainages [68–70]. Nowadays it is present in more than 1500 ha of the Ebro River Delta and the whole low Ebro, reaching more than 50 km upriver to the locality of *Miravet* [71].

These Ebro Delta specimens fall within the morphological variability and ecological range of *Pomacea maculata* (syn. *P. insularum*), and their mitochondrial DNA variation matched that of *P. maculata* [69]. However, posterior genetic studies found, not only *P. maculata* individuals but also *P. canaliculata* ones in the Ebro Delta apple snail invasion span [72].

The first actions taken by the authorities when Apple snail were first detected consisted in disseminating the risks of this invasion and alert the farmers, while searching methods to prevent the apple sail spreading and to encourage the farmers to destroy

eggs and snails. However, the measures taken were not effective and the infestation was impossible to eradicate. In fact, the 27th of July of 2010, the existence of an apple snail invasion was officially declared (*Resolució DOGC AAR/404/2010) from Generalitat de Catalunya)*. At the end of 2010 more than 300 fields (covering about 570 ha) had apple snail presence, and many rice plots had more than 100 individuals, which translated in huge losses for the farmers. The efforts on controlling its dispersal and the attempts to eradicate them were not successful [IRTA, personal communication].

The apple snail persisted burying itself during the winter and spreading to the river and the shores of *Deltebre*. In November 2011, one year after the official declaration of the invasion, more than 840 ha were infested, but the combination of maintaining dry fields all over the winter (which reduces the snail activity) and the use of phytosanitary products allowed to evade harvest losses in some cases. The over costs, however, make it hard for the farmers to reach benefits, and the Apple Snail mortality was between 65-85%. [IRTA, personal communication], therefore eradication was not achieved.

For the 2012 season, the authorities allowed to test a new treatment to eradicate the apple snail: flooding of fields and canals with seawater during 15 days in autumn. More than 600 ha were treated and the mortality was in all fields over 90%. Some fields even reached 100% of mortality and that coupled with preventing the re-infestation with appropriate infrastructure, achieved complete eradication in some fields. Nevertheless, the invasion continues and the risk of spreading to other regions is increasing.

The seawater treatments were repeated in 2013 and 2014, but the farmers began to complain since the residual salinity affected next year yield and neither the control nor the eradication were progressing [74]. The apple snail invasion received attention not only from the local government, but also from the Spanish state and the EU community. Between 2010-2017 more than 9 million euros were invested in control and eradication of the apple snail without the expected success (Table 1) [75].

Table 1. Inversion realized in Apple snail control and eradication by different authorities (period 2010-2017)

Authority	Inversion
Generalitat de Catalunya (local)	4.715.941,24 €
Gobierno de España (State)	1.891.963,61 €
European Union (EU community)	2.420.049,93 €
Total	9.027.953,42 €

From 2014 to nowadays, the authorities, farmers and research centres have developed and implemented action plans faced to control and eradicate the apple snail such as hand removal and destruction of specimens, machinery disinfection, installation of barriers in water canals and drainages and seawater treatments of strongly infested fields. In addition to these direct actions, the investment in research is also remarkable. One example is the EC H2020 project called NEURICE leaded by the University of Barcelona, which aims to develop new salt-tolerant rice varieties to fight the apple snail via seawater treatments, and to protect the rice farming sector against the climate change.

1.5. Rice blast, an old known disease

i. The disease and the pathogen

Rice blast is a rice disease caused by the filamentous ascomycete *Pyricularia oryzae* (syn. *P. grisea*, *Magnaporthe oryzae, M. grisea*). The symptoms of the infection include lesions or spots in all parts of the plant such as leaves, panicles, the culm or even the seeds (Figure 12). It is typically detected by the presence of rhomboid ash grey like spots. As it progresses, the spots can extend all along the leaf and destroy them. When the infection is severe and appears in crop's early stages, it can cause delays in development, flowering and maturation.

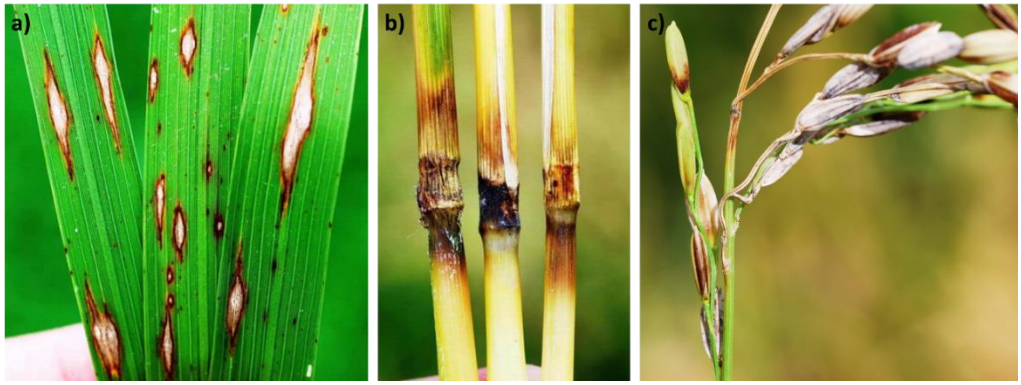

Figure 12. Rice blast (*P. oryzae*) lesions on a) leaf, b) neck and c) panicle. Images belong to Donald Groth (Louisiana State University AgCenter, USDA Forest Service).

However, the most economic impact occurs when the infection affects the neck of the panicle. In this case it can totally abort the panicles Moreover, even if the plants overcome the infection and the grain reach maturity, the collected grain is likely to be chalky and have milling defects, reducing their price in market.

In winter, the rice blast survives as conidiophores and mycelium on the plant residues after the harvest or in the ground. In spring, the temperature and relative humidity increase displays the mycelium sporulation producing conidiophores which are the primary source for infection. However, *P. oryzae* can also be disseminated by infected rice seeds or can hibernate in winter cereals or other plants like *Cynodon dactilon, Phragmites communis, Sorghum halepense or Arundo donax* [76,77].

The rest of the cycle is usually described in leaf, but it is similar in other tissues (Figure 13). Conidia carried by the wind or rain drops are deposited over the rice leaf surface. The spore germinates forming the appressorium, a specialized structure that allows this ascomycete to penetrate the plants cells. Once the hypha has penetrated the cuticle, a primary mycelium ramificates forming new invasive hypha that extracts nutrients from the plant cells alive. With the progress of the infection, the initial biotrophic behavior changes towards a necrotrophic behavior [76,77].

The first symptoms are visible in the host plant four to five days after the conidia germination. *P. oryzae* sporulation can reach about 20.000 conidia per day, initiating a new infection cycle. However, the dissemination area is small and the spores are usually between 1-5 m from the source. The optimum conditions for the germination of conidia and most of the cycle are between 24-28 °C of temperature and 90-100% of relative humidity, but can be triggered at 20 °C [76–78].

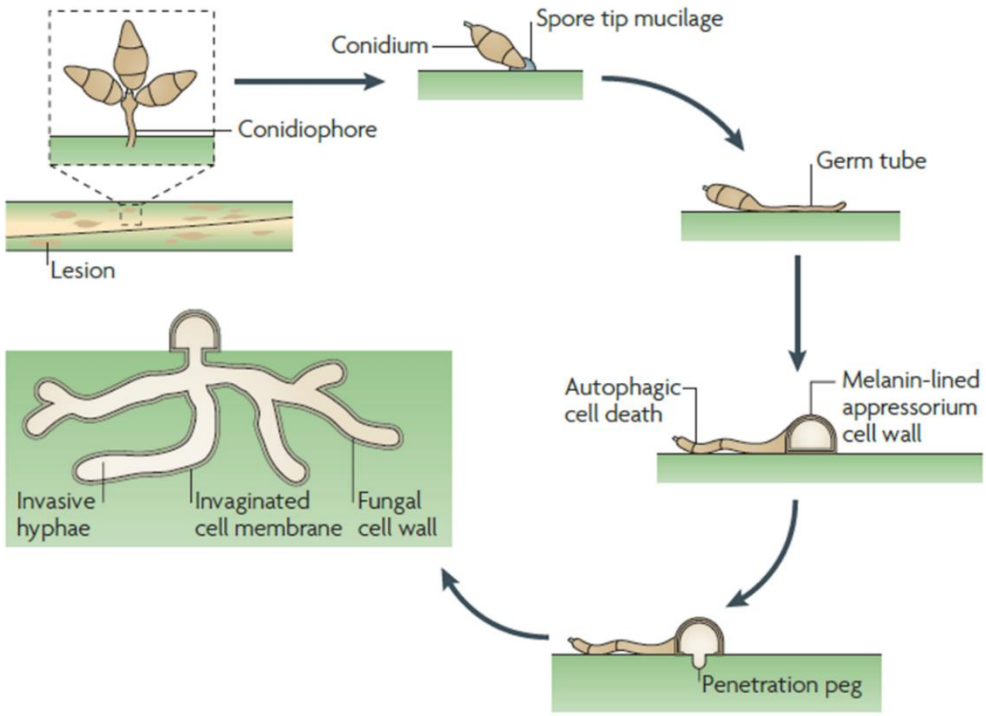

Figure 13. Life cycle of the rice blast fungus *Pyricularia oryzae*. The rice blast fungus starts its infection cycle when a three-celled conidium lands on the rice leaf surface. The spore attaches to the hydrophobic cuticle and germinates, producing a narrow germ tube, which subsequently flattens and hooks at its tip before differentiating into an appressorium. The single-celled appressorium matures and the three-celled conidium collapses and dies in a programmed process that requires autophagy. The appressorium becomes melanized and develops substantial turgor. This translates into physical force and a narrow penetration peg forms at the base, puncturing the cuticle and allowing entry into the rice epidermis. Plant tissue invasion occurs by means of bulbous, invasive hyphae that invaginate the rice plasma membrane and invade epidermal cells. Cell-to-cell movement can initially occur by plasmodesmata. Disease lesions occur between 72 and 96 hours after infection and sporulation occurs under humid conditions; aerial conidiophores with sympodially arrayed spores are carried to new host plants by dewdrop splash [77].

Rice blast is an old known disease around the world. In 1637, Soong Ying Shin described it in China and named it “rice fever”, in Japan it was identified in 1704 by Tsuchiya and in Italy Astolfi described a rice disease which matches with rice blast in 1828 [79–81]. Regarding Spain, in the earlier twentieth century great rice yield losses occurred being called “fallada”, and the described symptoms clearly match with the modern rice blast[82,83].

Nowadays rice blast is the most extended disease in the rice farmlands all over the world. The yield losses can rise to 50-90% in temperate and upland regions [79]. In Spain rice blast causes yield losses around 15-20% every year, even using specific fungicides, but in some areas like Valencia or Ebro river Delta blast losses can reach 50% [84,85]. Moreover, the use of fungicides increases the production costs reducing farmer’s profit.

The most common strategies to fight and control blast are the use of fungicides such as Tebuconazol 25% WG (Folicur ®) or Procloraz + Propiconazol 9% p/v EC (Bumper ®), the use of rice blast resistant varieties and specific blast-reducing farming practices [86]. However the recurrent use of the same fungicide active compound and the same resistant rice varieties promote the emergence of fungicide resistant blast strains, and new blast strains that overcome the resistance mechanisms of the rice varieties [76,77].

More than 100 blast resistance genes have been described in rice [87]. Most of them have been detected in South-East Asia or United States cultivars, but very little is described in Europe. For example, Jia et al. described the introgression of a large chromosome fragment around the rice blast resistance gene *Pi-ta* in elite cultivars. Among these cultivars, IR64 and Katy were analysed and showed a similar chromosome fragment, highly conserved, that includes *Pi-ta* gene among others. These two lines demonstrated to be blast resistant to different P. oryzae strains, in South-east Asia and U.S.A respectively. In Spain, only between 2000 and 2006, 27 virulence genes were detected in *P. oryzae* strains in Ebro River Delta, and different combinations of these

genes were detected every year, which makes difficult to obtain a high resistant variety [88]. Thus, a resistant variety is only resistant to blast during few years before the blast adaptation makes the main cultivated rice varieties to become sensitive. Consequently, the blast resistance mechanisms are rapidly surpassed by the fungus [89].

In the last decade, huge efforts are dedicated in the detection of blast resistance genes, in development of new molecular markers and in introducing resistance genes to commercial varieties [90–97]. One clear conclusion of these works is that the best way to fight *P. oryzae* is pyramiding genes, since the resistance rises for each added gene [98]. The solution in Spain probably goes through pyramiding a dozen of resistance genes because the large collection of *P. oryzae* strains. However, before that, some studies are needed about which genes are useful here and which are already surpassed, like the one from Jorge Perez [99].

1.6. Rice variety improvement

i. Classic breeding and backcross programs

The basis of classic breeding is defined as the selection of the best individuals among a population. The standard breeding techniques have been reviewed in various textbooks (e. g. Allard 1999 [100] and Acquaah 2012 [101]). In rice, the very first plats selections aimed to eliminate undesirable traits, like seed dehiscence and seed dormancy, but also to improve crop yields. Nowadays, classical breeding is referred to methods like the “pure line” selection of highly heterogeneous varieties, or the hybridization of two lines, varieties or landraces, and the posterior selection of the best individuals from the offspring by observing only the phenotype.

The classical breeding was the main crop improvement method until the end of World War II, when the world faced serious food shortages. At this point, in 1945 the United Nations Organization founded the Food Agriculture Organization (FAO), in order to attend the increase of food production by scientific means. This organization was in

charge of the collection and cataloguing of cultivar germplasm into varietal groups and the maintenance of indica and japonica germplasm, which proved a valuable and readily accessible source of variability for use by breeders all over the world [32,102].

A big germplasm collection allowed farmers to introgress, some desirable traits from foreign lines in their local varieties, such as increased yield, grain shape or pathogen resistance. Furthermore, the backcross programs were developed and thoroughly used in order to add a foreign desired trait into a local variety maintaining as much local traits background as possible. In a backcross scheme, after a first cross between the local variety (recurrent parent) and the foreign variety (donor parent), a series of crosses between the descendant and the recurrent parent are carried out in order to steadily replace the undesired traits from the donor parent by the local variety traits in the descendance. Each time a cross is performed, a fine selection of the descendants must be performed, to continue the breeding with those lines that are more similar to the recurrent parent and that have successfully inherited the desired trait [101].

The breeding until the '60s consisted mainly in the improvement of native landraces, the introgression of foreign desirable traits and the acclimatization of foreign varieties. Between 1930s and 1960s, the so called Green Revolution caused severe changes in the agriculture around the world. In this period of time new fertilizers, pesticides, herbicides and heavy farming machinery were developed, along with the high yielding varieties, which in most cases were semi-dwarf lines obtained after mutagenesis [32].

Mutagenesis, along with other techniques such as anther culture or double haploid production, the improvement of the polymerase chain reaction (PCR) and more recently, molecular markers and genetic modification, comprises what nowadays we call biotechnology-assisted breeding. These techniques, allows the fast generation of mutants, the generation of stable line from haploids or the selection and editing of genes or genotypes. Even that some of them began to appear during the Green Revolution, they were boosted with the advances in genomics along the '90s and in particular with the publication of the rice genome in 2002 [103].

The fully sequenced rice genome allowed scientist to detect genetic variations in germplasm collections, to develop DNA-based molecular markers, and to make genomic maps. These markers have been used to identify new genes and loci controlling traits of economic importance and have open the path to predict phenotypes from genotypes. Plant breeders have applied these markers in their breeding techniques, improving the precision and efficiency of the introgression of new characters and the development of new varieties [48,104–106].

Nowadays the genomic-assisted breeding has been successfully applied in several cereals [107–109] and new technologies such as CRISPR/cas9 (clustered regularly interspaced short palindromic repeats) and next-generation sequencing are being developed and applied to plant breeding [48,110].

ii. Genomic-assisted breeding (KASP technology)

The H2020 NEURICE project relies in a genomic assisted breeding scheme to fasten the introgression of the *Saltol* QTL in local varieties. The specific technology used is KASP, a reliable genotyping technology that uses competitive allelic-specific PCR. KASP technology is a patented commercial technique that provides high accuracy when genotyping with high flexibility in the primer design and thermal cycles [111–113].

KASP uses three components: (i) test DNA with the SNP of interest; (ii) KASP Assay Mix containing two different, allele-specific, competing forward primers with unique tail sequences and one reverse primer; and (iii) the KASP Master mix containing FRET cassette plus *Taq* DNA polymerase in an optimised buffer solution. In the first round of PCR, one of the allele-specific primers matches the target SNP and, with the common reverse primer, amplifies the target region. The Reverse primer binds, elongates and makes a complimentary copy of the allele-1 tail. In further rounds of PCR, levels of allele- specific tail increase. The Fluor labelled part of the FRET cassette is complementary to new tail sequences and binds, releasing the fluor from the quencher to generate a fluorescent signal (Figure 14) [111–113].

KASP technique can be easily carried out with specific chips (from 48 to 1536 well PCR-plates) where components are mixed, and the extracted DNA is placed. Reaction occurs in a thermal cycler and later fluorescence is read and digitalized. Analyses of the cluster allows to classify each marker as homozygous or heterozygous according to their fluorescence [111–113].

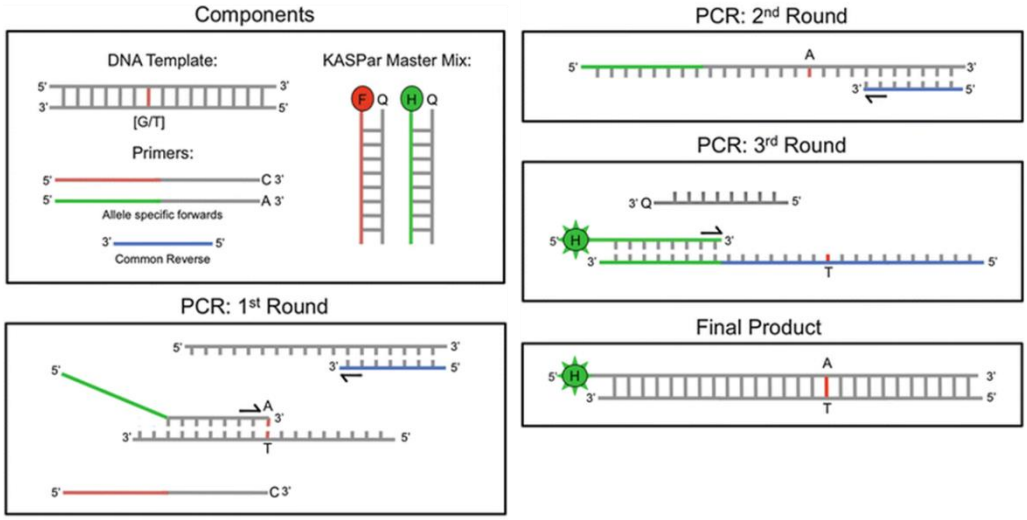

Figure 14. Diagram detailing the KASP genotyping chemistry. Components consist of: three user-designed primers (two allele-specific forwards and one common reverse) unique to a single SNP, two universals secondary oligos with attached 5' fluorophore and bound quenchers (included in KASP reagent), and DNA template. In the first rounds of PCR, only the common reverse and the allele-specific primer that corresponds to the specific genotype of the DNA template hybridize and extend. In this first round of PCR, a 5' tail is incorporated into the PCR product. During the second cycle of PCR, the common reverse oligo binds the template made from the first round of PCR and extends producing a complement to the allele-specific 5' tail. In the third round of PCR, the secondary oligos with the attached fluorophore hybridize to the PCR produce releasing the fluorophore from its quencher and incorporating it into the final PCR product. As amplification continues additional fluorophores are released from their quenchers producing a strong allele-specific signal [114].

iii. Embryo rescue

The embryo rescue is an in vitro technique that consists in separating the embryo from the rest of the seed and cultivating it in a nutritive medium so it develops to a whole plant. The embryos can be rescued to improve the germination rate in inherently weak

embryos, to break seed dormancy, to recover hybrid plants from interspecific or intergeneric crosses and to shorten breeding cycles [115].

The first record is from 18th century, from the work of Charles Bonnet who excised embryos of *Phaseolus* and *Fagopyrum* and planted them in soil, observing the germination of dwarf plants. In 1890, Brown & Morris developed nutrient solutions for germinating embryos. Despite this, the use of embryo rescue as a systematic technique begun in the 20th century with the works of Hannig (1904), who cultured embryos of *Cruciferae* in a medium containing mineral salts and sugar, under aseptic conditions and obtained healthy plants [115]. During the 20th century plenty of researchers like Knudson, Dieterich or Raghavan developed this technique to study the stages and development of the embryo and its requirements. Other authors such as Laibach gone forward and exposed the opportunity to germinate interspecific hybrids using embryo rescue. Turkey's experiments (1933) on cherry embryo culture marks a milestone, and his medium and procedure are widely accepted and successfully applied to other crops [115].

Nowadays, embryo rescue is considered a biotechnology technique and is widely used in almost any species. Some examples, just from the last year are in cucumber [116], peach [117], *Trifolium* [118], avocado [119], wheat [120], *Brassica* [121], Jatropha & Ricinus [122], peony [123] and pine [124]. For rice, in 2016, Tanaka *et al.* published a simplified biotron rice breeding system, which involves embryo rescue of the crosses to shorten the cycle between generations [125], taking in account the one published by Onishi *et al* in 2011 [126].

AIMS

2. AIMS

The general aim of this Thesis is to develop new rice varieties (*O. sativa*) tolerant to salinity stress, to fight the apple snail and the climate change, resistant to rice blast (*P. oryzae*) and adapted to the Mediterranean region of Ebro River Delta.

The specific aims are:

- To introgress a **salt tolerance** QTL named *Saltol*, which grants a high salt tolerance in seedling stage, from Asiatic *indica* rice lines to *japonica* Mediterranean rice varieties, following a MABC scheme assisted by embryo rescue and confirming the salt tolerance of the obtained lines by hydroponics assays.

- To assay **blast resistance** rice varieties from Asia and U.S.A and their hybrids with local varieties, in order to find new sources of blast resistance in Ebro River Delta, where particular climatic conditions and *P. oryzae* strains are presents.

MATERIAL AND METHODS

3. MATERIAL AND METHODS

3.1. Plant materials

For the salt tolerance introgression, four rice (*O. sativa*) lines were used in the backcross program: two recurrent parents and two salt tolerance donors. The recurrent parental lines were two different *temperate japonica* varieties, coded as PL12 (long grain) and PM37 (short grain), provided by *Càmara Arrossera del Montsià SCCL* (Amposta, España). This two varieties, commercialized by the SME, are elite varieties, well adapted to the Ebro River Delta and salt sensitive.

The salt tolerance donors were two *indica* lines from South East Asia, FL478 and IR64-Saltol, both long grained rice, provided by International Rice Research Institute (IRRI, Los Baños, Philippines) (Figure 15). FL478 is a RIL derived from the cross IR29xPokkali as commented in introduction and has a high salt tolerance. IR64-Saltol is a line derived from the cross IR64xFl478, having a moderate sat tolerance but higher agronomic and commercial interest than FL478.

Plants were grown in a greenhouse, of the *Servei de Camps Experimentals* de la Universitat de Barcelona, in 4 litres-pots filled with rice substrate. This rice substrate consists in a mix of 2:1 v/v of peat moss (Floratorf peat moss, Floragard Vertriebs, Oldenburg) and vermiculite (3 mm grain size), supplemented with controlled-release fertilizer [Osmocote Exact (15 + 9 + 11 + 2 MgO + micronutrients), The Scotts Company LLC, USA] at the rate of 2 $g \cdot L^{-1}$ of substrate. Additionally, $CaCO_3$ is added at 1 $g \cdot L^{-1}$ of peat moss, to adjust the substrate pH around 6.

Greenhouse temperature ranged from 20°C to 35°C, and the humidity ranges from 50% to 100%. Plants were irrigated daily automatically and fertilized two times a week manually with rice fertiliser (Table 2).

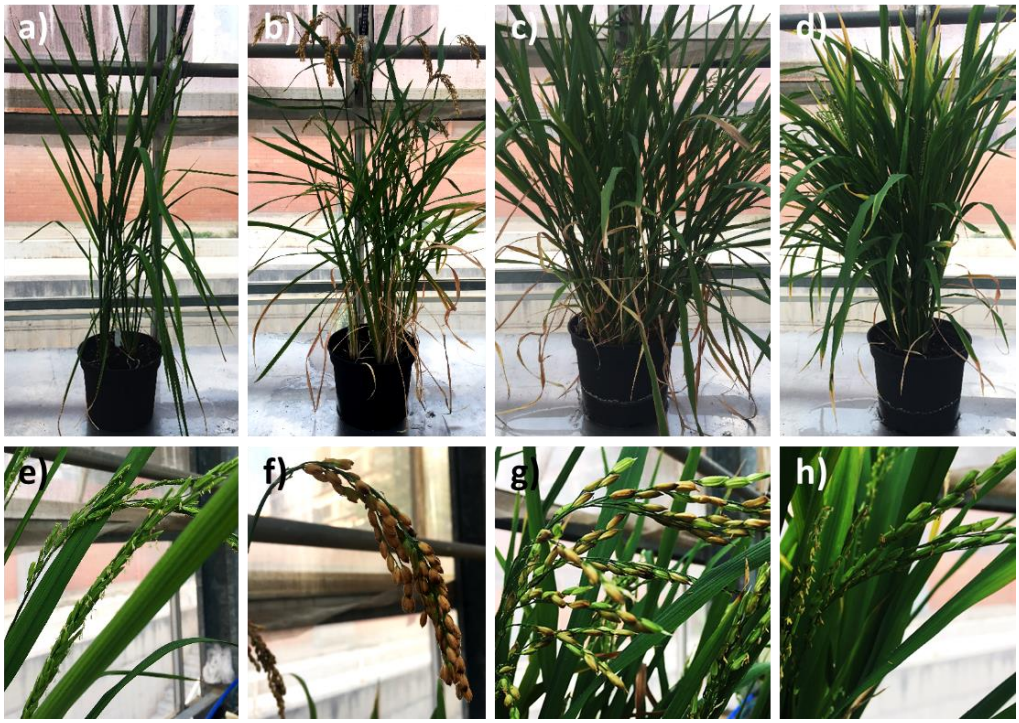

Figure 15. Images from lines used in the *Saltol* introgression and detail of the panicles (below each line): a,e) PL12, b,f) PM37, c,g) FL478 and d,h) IR64-Saltol.

Table 2. Composition for 5L of 40x Rice growing fertilizer.

40x Rice Growing fertilizer	
$NH_4H_2PO_4$	15.5 g
K_2SO_4	12.5 g
NO_3NH_4	12.8 g
$(NH_4)_2SO_4$	108.4 g
Urea	4.2 g
EDDHA-Fe 6%	3.1 g
Microelements	3.0 g
Distilled water	5 L

For blast resistance field assays in 2016 six candidates to be blast tolerant were tested, five from U.S.A: Bluebelle, M202-Katy21, 303011, 303012, 303013 and one from Philippines: IR64-Saltol. Also, one local variety, Montsianell, was assayed as blast susceptible control. Additionally, some F_2 hybrids between this foreign varieties and local ones were tested: OlesaxBluebelle (and the reciprocal), 303012xBluebelle, Bluebellex303013, M202-Katy21xMontsianell (and the reciprocal) and 303011xMontsianell. These F_2 hybrids were generated the year 2015 in greenhouse, following the forced hybridization method explained in this work and then self-pollinated one time.

For 2017 assays, the same foreign parental lines were tested with the exception of 303011 and IR64-Saltol, while Olesa was included. F_3 hybrids from the plants selected the year before were also tested, except for the cross M202-Katy21xMontsianell.

3.2. Experimental design

For the introgression of the salt tolerance:

a. Find and obtain salt tolerant rice (*O. sativa*) lines that carry the *Saltol* QTL.
b. Cross the salt tolerant rice lines with local lines by forced hybridization following a backcross scheme (5 generations).
c. Apply an embryo rescue protocol to speed up the breeding process and reach a BC_3F_3 in less than 3 years.
d. Analyse all the obtained plants each generation by PCR of an SSR marker inside the *Saltol QTL* to select those plants carrying the *Saltol* QTL.
e. Analyse the genetic background of the plants that inherited the *Saltol QTL* using KASP technology, to select those plants with the highest return to recurrent parent.
f. Confirm the salt tolerance of the obtained lines by hydroponic assays under salt conditions (80mM NaCl) evaluating their SES score, relative chlorophyll content, fresh weight and plant length.

For the blast resistance evaluation:

a. Find and obtain blast resistance rice (*O. sativa*) lines that carry some of the blast resistance genes described in the bibliography.
b. Cross the blast resistance rice lines with local lines by forced hybridization and self-pollination.
c. Evaluate the blast resistance lines and the hybrids obtained in field assays in the Ebro River Delta to determine their blast resistance against Mediterranean *P. oryzae* strains.

3.3. *Saltol* introgression

i. Marker Assisted Backcross scheme

To introgress the *Saltol* QTL, a marker assisted backcross (MABC) method was performed, following a typical scheme which involved the initial cross, three backcrosses (BC) and two selfing generations (Figure 16). During the backcrosses, the scheme was coupled to an embryo rescue (ER) technique to speed up the process. PL12 and PM37 were used as female recurrent parents, while FL478 and IR64-Saltol as male donors. Each cross was resumed as follows to simplify the work:

PL12 x FL478:	LP	PM37 x FL478:	MP
PL12 x IR64-Saltol:	LS	PM37 x IR64-Saltol:	MS

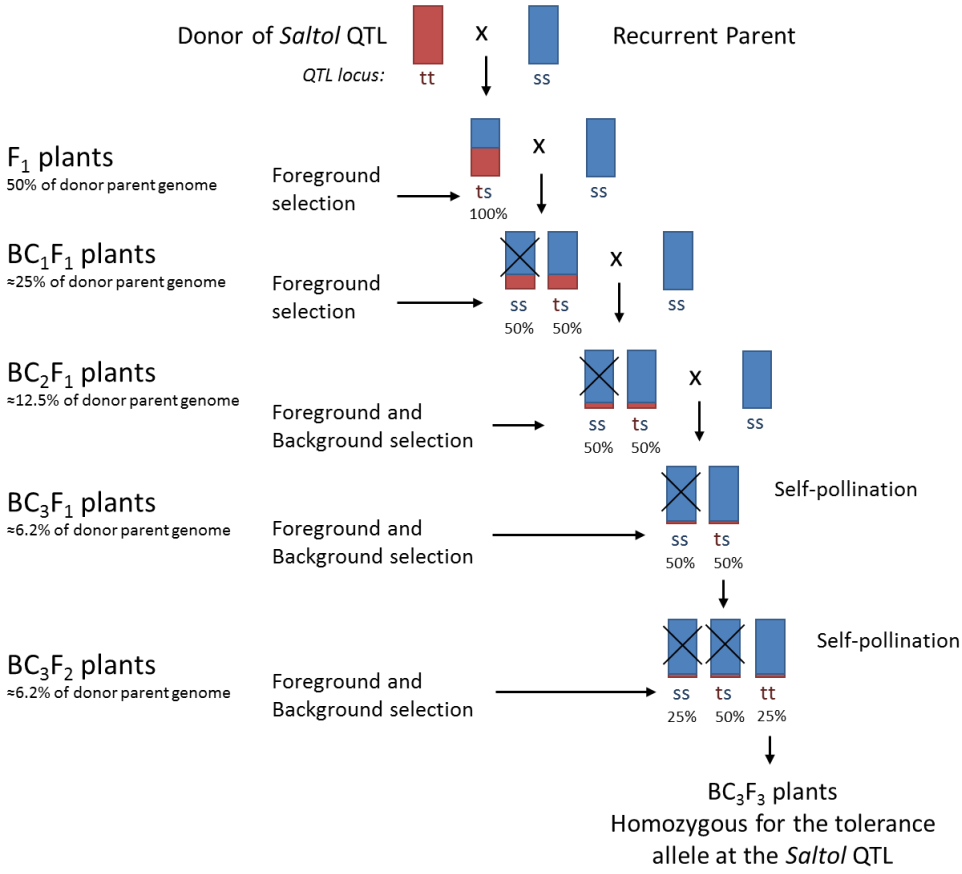

Figure 16. Molecular Assisted Backcross (MABC) scheme followed in this work. Colored boxes represent plant genome (blue for the japonica genome and red for the *indica* genome). In *QTL* locus, t refers to the tolerant allele while s to the susceptible one. Foreground selection was performed with a simple PCR targeting the center of the *Saltol* QTL. Background selection was carried out with KASP analyses.

In each generation, those plants presenting the introgressed Saltol QTL were selected (foreground selection), and from them, those having the highest return to the recurrent parent were also selected (background selection). The self-pollination generations enabled to fix the target chromosomal segment and multiply seeds for further experiments. At each generation the foreground selection was conducted with a simple SSR marker and then for the generations BC_2F_1 to BC_3F_2, a background selection was performed also (Figure 16). To optimize the use of resources, a two-step process was chosen for the background selection: a pre-screening of all plants using two markers in

the target zone and a higher density genotyping of the selected plants using chips (48 markers x 48 individuals- or 96 markers x 96 individuals-chips).

The workflow followed in each generation was: i) a forced hybridization, ii) embryo rescue of the seed, iii) a foreground selection with a simple PCR to select plants that introgressed *Saltol* QTL and iv) a background selection in two steps to select those plants with higher return to recurrent parent.

ii. Forced hybridization

To obtain the crosses, a forced hybridization was performed, since rice is an autogamous plant. A panicle in development was selected from a recurrent parent. The top and bottom flowers were cut off (the open ones and the immature) and only those unopened flowers with the anthers for about the middle of the flower's height were emasculated. The emasculation was performed cutting the top of the flower with scissors to be able to have access the anthers, which were sucked out using a vacuum pump coupled to a micropipette tip. The emasculated panicles were covered with a paper bag and labelled in order to avoid any undesired cross-pollination. All the process can be seen in Figure 17

At midday, when anthesis of the donor parental occurred (the panicles flowers open and the anthers carrying the fertile pollen came out), one panicle of the desired pollen-donor parental was introduced and shacked inside the paper bag containing the emasculated pollen acceptor parental panicle, releasing the pollen over the emasculated flowers. Once pollinized, the pollen-acceptor line panicle was covered again with the paper bag.

The pollinized panicles were allowed ten days to develop until milky stage, and then the paper bags were retired and the embryo rescue technique was performed over the obtained seeds.

At least 50 panicles were pollinated for each cross and generation to ensure a proper number of seeds. Pollinated panicles and obtained seeds were recorded, and the efficiency of the hybridization for each cross was calculated following the formula:

Efficiency = Obtained seeds / Pollinated panicles

Figure 17. Forced hybridization process: a) the top of the flower is cat, b) anthers are sucked with a vacuum pump coupled to a micropipette tip, c) emasculated flowers are covered with a paper bag till fertilization, d) when donor flowers enters anthesis their flowers opens and pollen can be used to pollinate emasculated flowers, e) after fertilization, the panicles are covered again with a paper bag and let to mature, f) after 10 days from fertilization, paper bag is retired an obtained seeds (black arrows) are counted.

iii. Embryo Rescue

The embryos were isolated from the rice seeds and grown posteriorly in *in vitro* medium following and adapting the embryo rescue method from Ohnishi et al. [126]. Immature seeds at 10 days after pollination were dehusked using a pair of tweezers and sterilized in 70% of ethanol for 1 min, followed by 30 min in a solution containing

2.5% sodium hypochlorite solution supplemented with Tween 20 (8 drops/L). Then, seeds were washed five times in sterile water under sterile conditions in a flow cabinet.

Seeds were placed on sterile filter papers under a binocular microscope and their seed coats were removed using a surgical knife and a pair of tweezers previously sterilized. Carefully, the embryo was removed with the surgical knife and was placed in jars or tubes containing embryo rescue medium, consisting in 0.5 MS salts (Murashige and Skoog Plant Salt Mixture), 0.025% MES monohydrate, 2% sucrose and 0.25% Gelrite, pH adjusted to 5.8 (all components from Ducheffa Biochemmie). All the process is described in Figure 18.

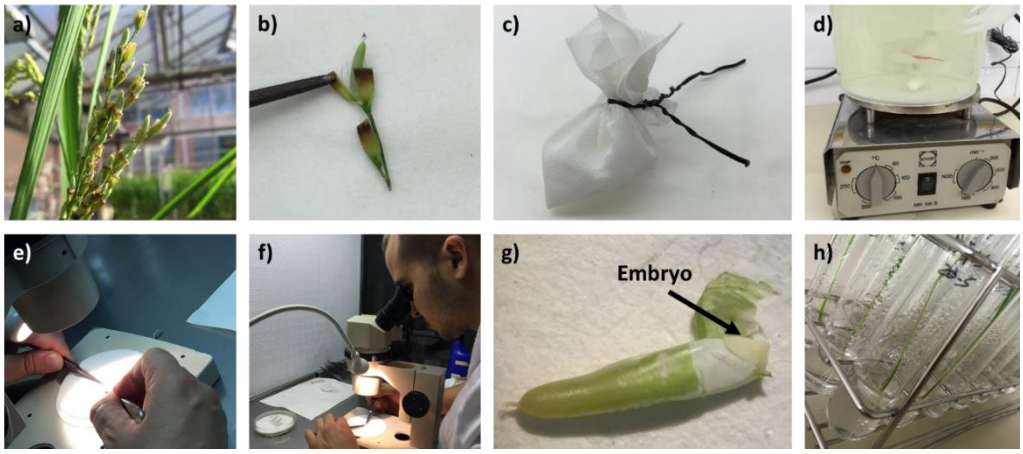

Figure 18. Embryo rescue protocol: a) seeds obtained 10 days after pollination, b) seeds are dehusked with tweezers, c) dehusked seeds are placed in a nylon or metallic mesh and it is closed with a wire, d) seeds are disinfected with sodium hypochlorite and Tween20, e) dissection of embryos is performed under a binocular glass magnifier, f) image of the general accommodation of material on the flow cabinet to secure the maximum sterility in the process, g) rice embryos are localized in one end of the seed, h) image of germinated embryos in MS medium 10 days after embryo rescue.

Jars with rescued embryos were sealed with Parafilm™ (Bemis Company, Neenah, U.S.A.) and transferred to a growth room (constant temperature of 24 ± 2 °C and 16h of light at 50-70 $\mu mol \cdot m^{-1} \cdot s^{-1}$) for 20 days. Then plantlets were acclimatized under greenhouse conditions in multi-pot trays filled with the previously described rice substrate.

A preliminary test was performed to confirm the reduction in life cycle described by Ohnishi et al. [126]. The embryos from 30 F1-LP seeds were rescued and the time from cross to flowering was averaged and compared with 30 more seeds from the same cross that hadn't followed the embryo rescue process, being sown once naturally maturated in the greenhouse.

Seeds from the F_1 and BC_1 crosses (LP, MP, LS and MS) were pooled together since no background selection was applied at those stages. From BC_2 onwards, the descendants from individual plants were identified separately, since the background selection allowed identifying differences in introgressed fragments and the return to recurrent parent between individuals.

iv. Foreground genotyping

The foreground selection was based in an SSR marker named SKC10 reported by Thomson [56]. This marker allowed us to detect which plantlets had inherited the *Saltol* QTL (at least the central part of it). The followed DNA extraction was an adaptation from Murray and Thompson CTAB protocol substituting Cetyltrimethylammonium bromide (CTAB) for Alkyltrimethylammonium bromide (MATAB).

About 100 mg of young rice leaves were collected in centrifugation tubes (Deltalab, Rubí, Spain) frozen in liquid N_2 and grinded using a Mixer Mill MM 400 (Retsch, Haan, Germany). This material was incubated for 1h at 74°C with 600 µL of MATAB extraction buffer (100 mM of Tris-HCL pH 8, 1.4 M NaCl, 20 mM EDTA, 2% MATAB, 1% PEG6000 and 0.5% Sodium sulfite), shaken every 15 minutes and cooled at room temperature. After that, 600 µL of chloroform: isoamyl alcohol (Chl:IAA, 24:1, v:v) was added and the tubes were centrifugated 5 minutes at 13,000 rpm. The supernatant was transferred to new tubes, where 5 µL of RNAse A (10 mg/ml) was added, and incubated for 30 minutes at 37°C. Following that incubation, 1 mL of Chl:IAA (24:1, v:v) was added and the tubes were centrifuged again for 5 minutes at 13,000 rpm, and the supernatants was

transferred to new tubes. Then, 600 µL of isopropanol was added to these tubes and they were centrifuged for 5 minutes at 13,000 rpm. The supernatant was discarded and 400 µL of ethanol 70% was added. One last centrifugation for 5 minutes at 13,000 rpm was performed and the supernatant was discarded. The tubes containing the DNA pellet were left to dry overnight (over a clean surface, upside down). Next day the DNA was re-suspended in 50 µL of Ultra High Quality sterile water and slowly shacked for 5 minutes in an orbital shaker. All components were purchased at Sygma-Aldrich (San Luis, USA) and the centrifuge used was a Digicen 21R (Ortoalresa, Madrid, Spain).

The PCR protocol was adapted for the polymerase requirements. Each reaction of 20 µL consisted in 2 µL 10x Paq-5000 Reaction Buffer, 0.4 µL $MgCl_2$ 25mM, 0.4 µL dNTP Mix (10mM each), 0.4 µL Forward primer 10 mM (ATAGGGGATATTGGCTGCAC), 0.4 µL Reverse primer 10mM (CAACCAAGCGTGACTAAAAAGA), 2 µL Enhancer (4M betaine, 10mM Dithiothreitol and 10% Dimethylsulfoxide), 0.2 µL Paq-5000 DNA Polymerase, 2 µL DNA and 12.2 µL H_2O. All products were from Werfen (Barcelona, Spain) except Paq-5000 DNA Polymerase and Reaction Buffer (Agilent Technologies, Santa Clara, U.S.A.) and primers (Laboratorios Conda, Madrid, Spain).

The thermocycler used was a Verity Thermal Cycler from Applied Biosystems (Foster city, U.S.A.) and the program consisted in an initial denaturation of 5 minutes at 94°C, followed by 30 cycles of 45 seconds at 94°C for denaturation, 45 seconds at 55 °C for annealing and 30 seconds at 72°C for synthetize DNA. Finally, after 30 cycles, 7 minutes at 72°C for complete DNA synthetization.

A gel electrophoresis was performed to analyze the DNA amplification. Gels contained 3% agarose in TAE buffer (40 mM tris(hydroxymethyl)aminomethane, 20 mM acetic acid glacial and 1 mM ethylenediaminetetraacetic acid in Milli-Q water). The DNA was dyed using *GelRed* (Biotium, Fremont, U.S.A.). The amplified DNA ran in the agarose gel for 1h before reveling it under ultraviolet light.

v. Background genotyping

The chosen genotyping technology was KASP (Kompetitive Allele Specific PCR) since it is a fast method, reliable and requires only a very small quantity of DNA. With this technique, SNP alleles are labeled with different fluorescent dyes. For individual markers (pre-selection), the genotyping was performed using LC480 qPCR machines for two flanking markers from Thomson's work (RM10694 and RM 10793). For chips, the genotyping was performed with Fluidigm assays on BioMark HD machines. About 240 KASP markers taken from the list of 2000 validated rice markers established by the Integrated Breeding platform (https://www.integratedbreeding.net) were tested in Montpellier by CIRAD on the 4 parental lines used and sets of 48 polymorphic markers were determined for each cross.

KASP analyses were performed in the Centre for Research in Agricultural Genomics (CRAG, Bellaterra, Barcelona), by Mireia Bundó, under the supervision of Blanca San Segundo. They developed new SNPs markers for these specific crosses to a final number of 68 markers, distributed along the 12 rice chromosomes with a density of 3 to 7 markers/chromosome with the exception of the *Saltol* QTL carrier chromosome, which was more saturated, having up to 11 markers, four of them inside the *Saltol* QTL region.

3.4. Hydroponic salt tolerance screening

Hydroponics assays were conducted under greenhouse conditions in the *Servei de Camps Experimentals de la UB (SCE).* Three experiments were realised, each one using 18 different BC_3F_3-LP genotypes plus the parental lines (PL12 and FL478). The design was an adaptation from the standard hydroponic evaluation in rice from the International Rice Research Institute (IRRI, Philippines) with a modified Yoshida solution (Yoshida *et al*., in 1976).

Each experiment consisted in three plastic trays with NaCl treatment (80 mM) and three control trays (1 mM), each one with 6 genotypes (plus the 2 parental lines) and 5 replicates, distributed in a randomized design (Figure 19). Perforated Styrofoam and foam pieces were used to support the seedlings as described in Figure 20. Each assay consisted in three phases or periods: water, Yoshida and Yoshida+NaCl (Figure 21).

	A	B	C	D	E	F	G	H
1	LP-2	LP-5	LP-2	LP-1	LP-2	PL12	FL478	LP-6
2	LP-1	LP-1	LP-6	FL478	LP-6	PL12	LP-3	LP-1
3	LP-3	LP-5	LP-2	LP-3	FL478	LP-3	LP-1	FL478
4	PL12	LP-4	PL12	LP-4	LP-4	LP-5	LP-2	PL12
5	LP-5	FL478	LP-4	LP-6	LP-3	LP-4	LP-6	LP-5

Figure 19. Schematic representation of the randomized plant distribution in a hydroponic tray. In each tray, 5 replicates of 6 lines and the 2 parental lines (PL12 and FL478) were randomly distributed.

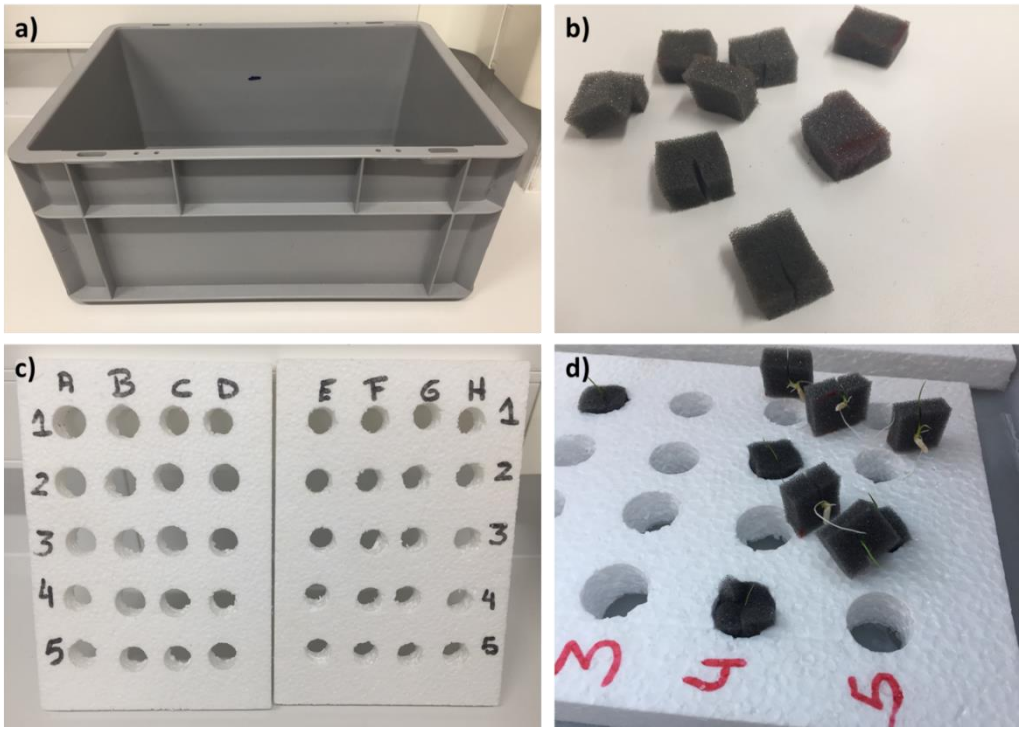

Figure 20. Material used in hydroponics: a) plastic tray of 10L, b) foam with a small cut to support seedlings, c) perforated Styrofoam and d) assembly of foam and seedlings on the Styrofoam.

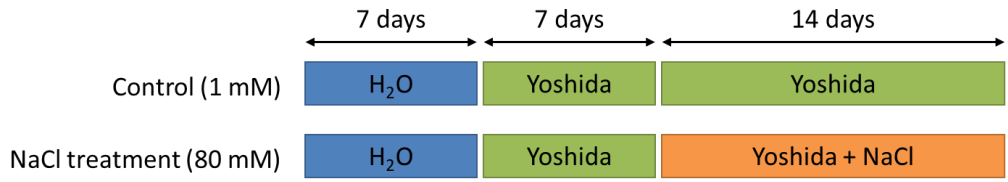

Figure 21. Schematic representation of the different periods and duration of the hydroponic assay. The first H2O phase was performed in petri dishes, while the Yoshida phases were in the plastic trays. Solution was renewed every 7 days and pH and EC was monitored every 2 days. The evaluation of plants was performed at the end of the 14 days Yoshida/Yoshida + NaCl period.

For the H2O phase, seeds used for hydroponics assays were surface-disinfected using 70% ethanol for 3 minutes, followed by a 30-minute disinfection using sodic hypochlorite 30% solution supplemented with Tween 20 (at 0.02%). Disinfected seeds were rinsed 5 times using sterile water in a laminar flow cabinet. The disinfected seeds were sown in sterile petri dishes containing a sterile filter paper with 2 ml of sterile distilled water. The plates were maintained at 28ºC with a 16h/8h light/darkness photoperiod (50-70 $\mu mol \cdot s^{-1} \cdot m^{-2}$) for one week. A total of 25 seeds were disinfected and sowed for each line and experiment (50 seeds for the parental lines) to have a surplus of plantlets to be able to make a homogenous selection in the Yoshida phase.

Once seeds germinated, seven days after sowing, each container was filled with 10 litres of Yoshida solution (Table 3) and placed in the greenhouse. The plate-germinated plantlets were placed in the middle cut of the foam pieces just where root starts. Then, the foams with the seedlings were placed in the correct well of the perforated Styrofoam, following the randomized design and ensuring that the root was in contact with the nutrient solution.

Table 3. Modified Yoshida solution final concentrations and Stock solutions. One liter of each macronutrient stock solution was prepared (mixing both phosphorous reagents). Micronutrients were prepared mixed in 500 mL except iron, which was prepared apart [127].

	Element	Reagent	Final concentration	Stock Solutions	
Macronutrients	N	NH_4NO_3	1.43 mM	100x	11.43 g/L
	S	K_2SO_4	0.51 mM	100x	8.94 g/L
	P	KH_2PO_4	0.85 mM	100x	11.55 g/L
		K_2HPO_4	0.12 mM	100x	2.14 g/L
	Ca	$CaCl_2 \cdot 2\ H_2O$	0.75 mM	100x	11.08 g/L
	Mg	$MgSO_4 \cdot 7\ H_2O$	1.64 mM	100x	40.52 g/L
Micronutrients	Fe	Na_2EDTA	37.75 µM	20 mM	7.44 g/L
		$FeSO_4 \cdot 7\ H_2O$			5.56 g/L
	Mn	$MnCl_2 \cdot 4\ H_2O$	9.5 µM	10,000x	1.88 g/0,1L
	Mo	$(NH_4)_6Mo_7O_{24} \cdot 4\ H_2O$	0.08 µM		0.09 g/0,1L
	B	H_3BO_3	18.89 µM		1.17 g/0,1L
	Zn	$ZnSO_4 \cdot 7\ H_2O$	0.15 µM		0.04 g/0,1L
	Cu	$CuSO_4 \cdot 5\ H_2O$	0.15 µM		0.04 g/0,1L

After one week growing in Yoshida solution, plants were subjected to the NaCl treatment (fresh Yoshida medium containing the desired NaCl concentration). The Yoshida solution was renewed once per week and pH and EC was controlled every two days with a hand pH-meter and conductivity-meter and adjusted to pH 5 and the corresponding EC, 1 mM or 80 mM, when necessary.

The standard evaluation system (SES) for salt injury developed in the IRRI in 2002 was used to determine the salinity tolerance of the plants [128]. This system assigns an injury score based on the plant growth and the appearance of salt stress symptoms (Table 4). The evaluation was done at the end of the salt treatment (14 days after the salt addition). We standardized the results to detect in an easiest way those lines that performed similar or better than FL478. The formula applied was:

Standardized SES = Salinity SES – Control SES

Table 4. Standard evaluation system for salt injury in rice, adapted from the IRRI [128].

Injury score		Description
1	Highly tolerant	Normal plant growth, only the old leaves show white tips with no symptoms on young leaves
3	Tolerant	Near normal growth, but only leaf tips burn, a few older leaves become partially whitish and rolled
5	Moderately tolerant	Growth severely retarded, most old leaves severely injured, a few young leaves elongating
7	Sensitive	Complete cessation of growth, most leaves dried, only few young leaves still green, some plants dying
9	Highly sensitive	Almost all plants dead or dying

Relative chlorophyll content was measured with an SPAD (Konica Minolta, Tokyo, Japan) after 14 days of salt treatment, on the last fully develop leaf. Three measures from each plant were performed, with a total of 15 measures per line. Fresh weight was determined with a precision scale, isolating shoot and root for each plant, and pooling together all the replicates from each line. Plants were photographed and plant length was measured later using the program ImageJ (National Institutes of Health, Bethesda, U.S.A.). The following formulas were applied to determine the reduction in fresh weight and plant length due to NaCl treatment:

$$\text{Fresh weight reduction (\%)} = ((FW_{control} - FW_{NaCl}) / FW_{control}) \times 100$$

$$\text{Length reduction (\%)} = ((Length_{control} - Length_{NaCl}) / Length_{control}) \times 100$$

Greenhouse temperature was recorded for the three experiments, being the mean 26.2 ± 2 for the first experiment, 23.5 ± 3 for the second and 24.2 ± 3 for the third. Daylight in greenhouse was supplemented with lights at a 16h/8h light/darkness photoperiod (50-70 $\mu mol \cdot s^{-1} \cdot m^{-2}$).

3.5. Field assays for rice blast resistance

Field assays were conducted in *Càmara Arrossera del Montsià* experimental fields, Amposta, in the Ebro River Delta. A selection of local and foreign rice lines and some hybrids between them (see 3.1. Plant materials) were assayed in 2016 and 2017 field trials.

The design consisted in rows of 30 hand-transplanted plants for each line or hybrid, with a separation of 25 cm between plants and 50 cm between rows and three replicates for each, randomly placed. Fertilization was carried both years following the local management, with 2/3 of total nitrogen in the form of urea directly on soil and 1/3 of the nitrogen in the form of ammoniumsulfate at panicle initiation stage, approximately, 100 units of nitrogen fertilizer (UNF) and 50 UNF respectively. An herbicide treatment was applied to the field before transplanting the lines. Additionally, the *Agrupació de Defensa Vegetal de l'arròs i altres cultius al Delta de l'Ebre* (ADV) yearly performs two treatments against rice stem borer using airplanes during the campaign. No fungicide treatment was applied, and weeds were retired by hand along the season.

A monthly evaluation was performed in July, August and September, taking into account homogeneity, tillering, height, cycle (following Lancashire et al. protocol [129]), stem borer affectation and rice blast affectation (Table 5). Some observations regarding extreme or interesting characters were recorded. By the end of September, the most interesting hybrids lines individuals were selected and their seed was harvested to perform the next field assay.

Table 5. Code used for the rice field evaluation and the correlation with each characteristic.

Code	Homogeneity	Tillering	Stem borer	Rice blast
+ + +	>90% homogenous	>40 tillers	0-1affected plants	0 - 0.5% leaf affectation
+ +	50 - 75% homogenous	20-40 tillers	2-5 affected plants	0,5 - 1% leaf affectation
+	25 - 50 % homogenous	10-20 tillers	5-10 affected plants	1 - 2% leaf affectation
-	<25% homogenous	<10 tillers	>10 affected plants	>2% leaf affectation

3.6. Statistics

All the data was analysed using the Statgraphic Centurion XVII software (Statpoint Technologies, Warrenton, U.S.A.). For the SPAD values, two ways ANOVA were performed for each experiment, to compare data for treatment (control/salinity) and for line factors. Bonferroni tests were followed for groups comparison. Regarding fresh weight and plant length, Chi-square tests were performed, comparing with FL478 values. Finally, for SES data, any test was powerful enough because data didn't follow a normal distribution neither variance was homoscedastic.

RESULTS AND DISCUSSION

4. RESULTS AND DISCUSSION

This work presents the first introgression of *Saltol* QTL in *temperate japonica* rice varieties. PL12 and PM37 are two elite varieties, adapted to the Ebro River Delta, which suffer from salinity stress in some coastal cultivated areas. Moreover, seawater treatment of infested fields can be determinant in order to eradicate the apple snail pest although causing a residual soil salinity increase. The discovery and characterization of the *Saltol* QTL opened the possibility to transfer salt tolerance at seedling stage from FL478 and IR64-Saltol to PL12 and PM37. Thus, the use of a MABC scheme on temperate japonica recurrent parental lines led to the obtaining of European salt-tolerant lines in a short time.

4.1. *Saltol* QTL introgression

i. Breeding efficiency

Between 50 and 100 panicles were emasculated and pollinated for each cross in each generation, but the efficiency, represented as the number of seeds per pollinated panicle, was not similar for each cross (Table 6). For the PL12 crosses, the forced breeding efficiency increased steadily generation BC1 to BC3 for the cross PL12xFL478 (from now on LP), as expected [131,132]. Contrary for the cross PL12xIR64-Saltol (from now on LS), the forced breeding efficiency decreased each generation due to the fixation of partial flower sterility coming from the most lines of this cross, where all the offspring showed about a 75% of panicle sterility. Because of that, after BC_3 the LS cross was discarded.

Regarding the PM37 crosses, both crosses displayed a good performance and showed an increase in seeds per cross. For the cross PM37xFL478 (from now on MP), more than 1,500 seeds were obtained. However, at the end we selected PM37xIR64-Saltol (from now on MS), to continue to self-pollination since more seeds were obtained from this

cross (more than 2000) and to ensure the presence of backcrossed lines from both salt-tolerance donor parental in field.

Even being feasible to perform more than one rice generation per year in temperate regions, a heated greenhouse or a growing chamber, with controlled conditions, is essential to increase the number of generations per year. Rice is strongly affected by temperatures below 20 ºC and poor light intensity and even with this infrastructure, production of seeds is much lower in cold months [133].

Table 6. Number of pollinated panicles, number of obtained seeds and average seed set per crossed panicle (efficiency) for each of the cross and backcross generations.

Generation	Cross	Pollinated panicles	Obtained seeds	Efficiency	Total seeds per generation
BC_1	PL12 x FL478	83	122	1.5	1456
	PL12 x IR64-*Saltol*	116	277	2.4	
	PM37 x FL478	105	250	2.4	
	PM37 x IR64-*Saltol*	178	807	4.5	
BC_2	PL12 x FL478	137	380	2.8	1459
	PL12 x IR64-*Saltol*	80	86	1.1	
	PM37 x FL478	51	550	10.8	
	PM37 x IR64-*Saltol*	69	443	6.4	
BC_3	PL12 x FL478	135	1611	11.9	5736
	PL12 x IR64-*Saltol*	79	58	0.7	
	PM37 x FL478	204	1776	8.7	
	PM37 x IR64-*Saltol*	219	2291	10.5	

We recorded the number of panicles pollinated and seeds obtained each month (Figure 22). In summer (June, July and August) the amount of obtained seeds is higher than in spring or autumn, while the seed production in winter is almost null. (Figure 22). As an example, in May 2016 the number of obtained seeds were much lower than in August same year, even with more pollinated panicles. Furthermore, a higher amount of seeds was obtained in May 2017 in comparison to May 2016 since in 2016 the mean temperature was lower than the year after. However, the temperature could not be the only factor affecting the breeding success between 2016 and 2017, since, in 2016 the performed crosses were principally F_1 and BC_1 generations, while in 2017 the crosses were mostly BC_2 and BC_3. As we have seen before in Table 6, the more advanced is the breeding, the more crossing compatibility is obtained between breeding lines and the recurrent parent. Furthermore, as we worked in a continuous way, generating hybrids, selecting them and progressing through generations, in each month different number of plants were ready to be donors (in flowering phase).

FL478 and other Pokkali derived lines have been used to transfer the *Saltol* QTL into elite varieties such as IR64, ADT45, BR11, BRRI, CR1009, Dhan28, Gayatri, MTU1010, PB1121, PR114, Pusa44 or Sarjoo52, among others [41,47,48,57,130] and some of them are being commercialized in South-East Asia for salt-affected areas [48]. However, all these lines are from the *indica* or *basmati* cultivars and it has never been attempted to introgress in Mediterranean temperate japonica varieties before.

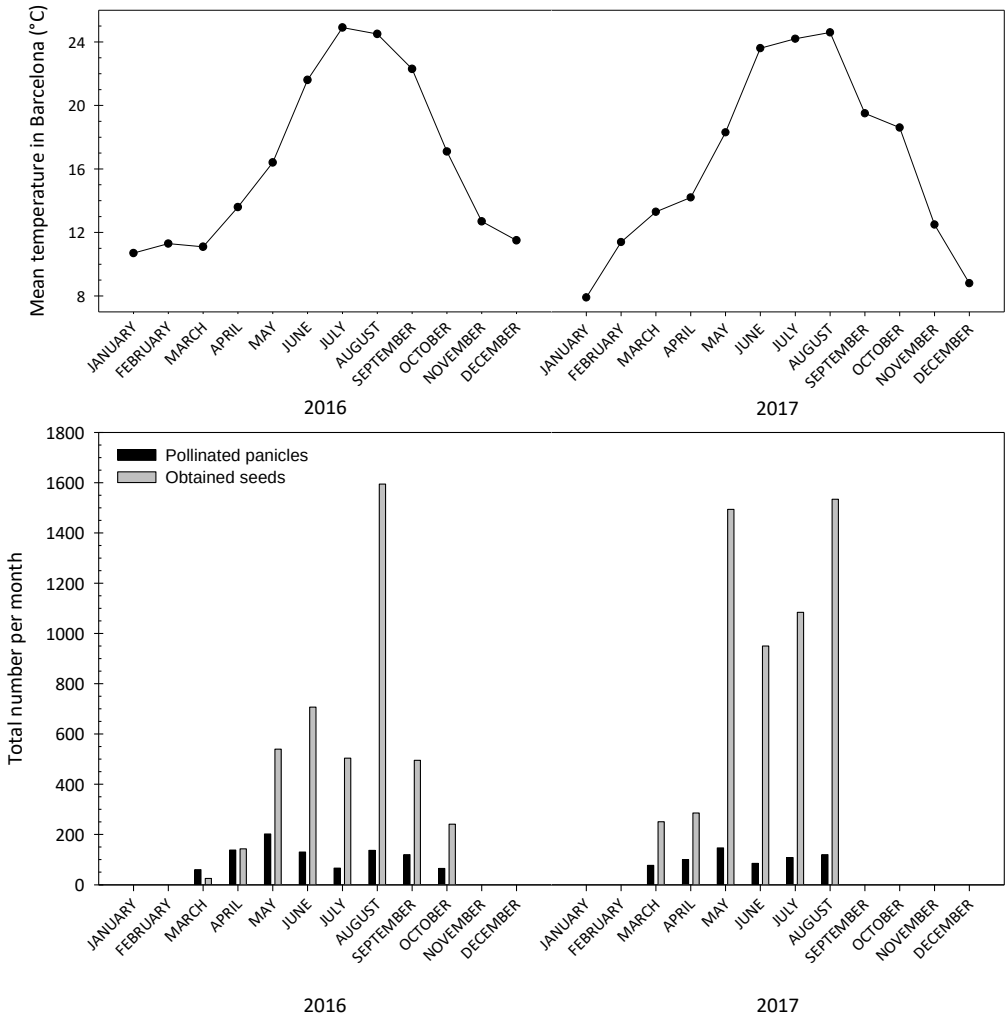

Figure 22. Mean temperature in Barcelona (°C) for each month during 2016 and 2017, obtained from the Fabra Observatory register [134]. Total number of Panicles pollinated and Obtained seeds per month during 2016 and 2017.

ii. Embryo rescue

In the preliminary test, the 30 immature seeds rescued embryos germinated *in vitro* were acclimatized to greenhouse as 3 leaves plantlets with just 20 days after the embryo rescue. On the other hand, seeds that were naturally matured in the panicle and sown, still needed 10 to 15 days to germinate. In conclusion, embryo rescued plants started flowering 30 days before the non-embryo rescued cross pollinated at the same time. Thus, we confirmed that the embryo rescue protocol allowed us to shorten 30 days the rice cycle as represented in Figure 23.

This cutback of the cycle is lower than the one described by Onishi et al. [126] and Tanaka et al. [125], although they use other lines, complementary methods further than embryo rescue (like CO_2 chambers), and do not perform a mass backcross scheme with thousands of plants. Even that, the application of such a technique allowed us to produce two generations per year, securing the BC_3F_3 generation in two and half years and ready to go to field assays in 2018.

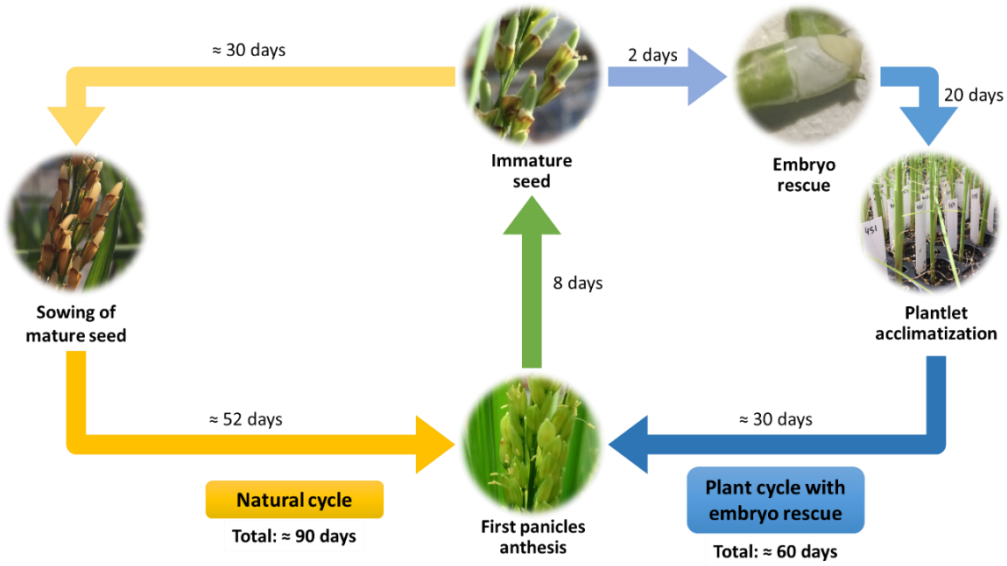

Figure 23. Schematic representation of rice cycle with embryo rescue compared to its natural cycle. A cutback of 30 days in cycle can be achieved using embryo rescue in 10 days immature seeds.

iii. Foreground analyses

The foreground analyses were based in a simple PCR and gel electrophoresis to detect a single SSR marker in the centre of the *Saltol* QTL. These analyses allowed us to select heterozygous plants in each backcross F1 generation, and homozygous *Saltol* plants after the BC_3 self-pollinations.

The SKC10 SSR marker selected which was first reported by Thomson[56], has two different alleles, one present in the FL478 and IR64-Saltol (salt tolerant) donor lines and a different allele in the Mediterranean japonica varieties, PL12 and PM37. The Saltol allele gives a 186bp PCR product, while the Mediterranean allele amplifies a 234bp fragment as shown in the Figure 24. The heterozygotes, from F_1 to BC_3F_1, showed both bands. Other authors use the SSR marker RM3412 also described in Thomson's work [57,130,135]. However, the resulting 110bp amplification was too small to be properly detected using agarose gels.

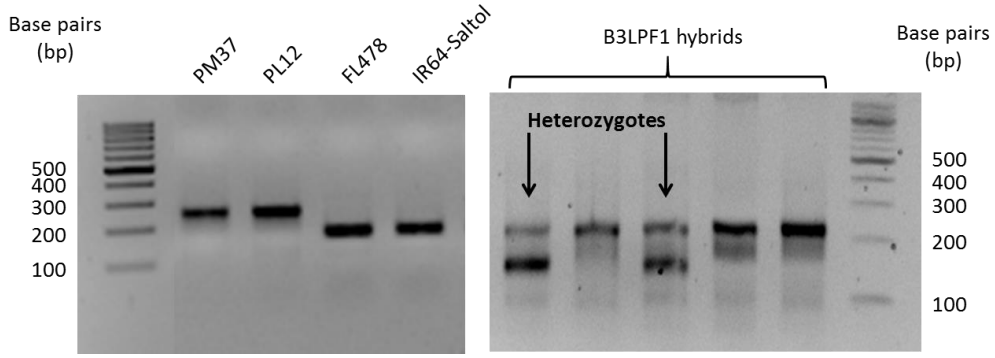

Figure 24. DNA amplifications gel electrophoresis of the SKC10 SSR alleles of the *Saltol* QTL (FL478 and IR64-Saltol, 186bp) and susceptible allele (PM37 and PL12, 234bp) on the left, and different BC_3LPF_1 heterozygote and 234bp allele homozygote lines on the right. Images kindly provided by M. Bundó, CRAG.

It was expected a 50% heritability of the *Saltol* salt-tolerance allele (t) for the backcross generations since a heterozygous *Saltol* tolerant/sensitive (t/s) was always used to pollinate the homozygous s/s local lines. Surprisingly, we have obtained lower heritability, between 25% and 40% due to undesired spontaneous self-pollinations.

Some parts of the anthers may get stuck in the emasculated flowers provoking self-pollinated and increasing the number of descendants with the s/s allele combination [108,132].

For each generation we analysed as much plants as possible (Table 7), with the aim of keeping as much different source lines for the next cross without provoking a big bottle neck. However, for the BC1 generation, we had problems with the technique, some products were contaminated and ultimately only about 20 plants per cross were finally analysed.

This relative low number of plants per cross in BC1 generation could lead to the fixation of a sterility gene in the LS cross, since from BC_1F_1, all the descendants were almost 75% sterile and we could not recover the fertile character in any case. Luckily, for the rest of the crosses this sterility problem did not occur.

For the rest of generations, as commented before, hundreds and thousands of plants were analysed in order to have as much SKC10 heterozygous plants as possible to ensure a strong background selection through the KASP chips.

Table 7. Rice plants (*O. sativa*) tested in each generation and cross for foreground PCR.
LP: PL12 x FL478 MP: PM37 x FL478
LS: PL12 x IR64-Saltol MS: PM37 x IR64-Saltol

	Number of plants tested			
	LP	LS	MP	MS
F_1	75	69	72	63
BC_1-F_1	22	14	24	18
BC_2-F_1	161	43	335	293
BC_3-F_1	448	45	211	1328
BC_3-F_2	615	X	X	1126

X: work stopped on these crosses

iv. Background analyses

The breeding process was performed in a continuous way, avoiding to stop crossing until the generation from all crosses was fulfilled. Consequently, different generations for the different crosses finally overlapped. This way the process was speeded up, although we ultimately had to coordinate with genotyping process. Thus, only a fraction of the generated plants could be analysed in each generation (Table 8).

In BC_1-F_1 generation a deeper scan of the QTL was also performed with two flanking markers for the selected plants for each cross. This preliminary analysis allowed us to select the best plants to be analysed by KASP and to be used for the next generation: those with the two flanking markers heterozygous, which had an increased probability to have introgressed the *Saltol* QTL intact. In the BC_1-F_1 generation, there was no selection because the low number of plants, but this recombination selection was further implemented in the rest of generations.

The first KASP analysis was performed on BC_2LS-F_1, BC_2MP-F_1 and BC_2MS-F_1 generations, allowing us to select 10 BC_2MS-F_1 plants with more than 75% recurrent parent genome (with some plants with a maximum of 78.8% recurrent parent genome). Although BC_2LS-F_1 and BC_2MP-F_1 plants were tested in this first KASP analysis, we decided to continue only with LP and MS, because the problems of sterility in LS cross and to have one population derived of each salt donor parental (explained in 4.1.i. Breeding efficiency). Thus, for the next generation plants from LS and MP were selected, but their progeny was not analysed by KASP.

Regarding LP crosses, the first KASP analysis was performed on BC_3LP-F_1 generation, but was performed simultaneously with the described above, because we already had this generation when the KASP analysis become available. In this generation, more than a 95% of return to the recurrent parent was achieved and 8 plants were selected to be self-pollinated. The next KASP analyses, performed on the BC_3LP-F_2 and the BC_3MS-F_1 plants, allowed us to select 16 and 12 plants respectively, reaching 98.2% and 95.2% of return to the recurrent parent genome.

The last KASP analysis was performed on BC_3MS-F_2 plants, and 10 plants between 95.2% and 98.2% of return to the recurrent parent genome were selected. All of the BC_3-F_2 homozygous tolerant *Saltol* plants were self-pollinated again to obtain the BC_3-F_3 seeds to be tested hydroponically for salt tolerance.

Table 8. Summary of rice (*O. sativa*) background genotyping and selection of rice lines from each of the 4 crosses at each backcross stage.
LP: PL12 x FL478 MP: PM37 x FL478
LS: PL12 x IR64-Saltol MS: PM37 x IR64-Saltol

	Number of plants tested and selected			
	LP	LS	MP	MS
BC_1-F_1 plants heterozygous at flanking markers	10	5	11	7
BC_1-F_1 selected plants and used in hybridization	10	5	11	7
BC_2-F_1 plants genotyped with KASP	n.d.	8	11	58
BC_2-F_1 plants selected and used in hybridization	n.d.	5	8	10
% recovery recurrent parent (max.)	n.d.	75.9%	72.4%	78.8%
BC_3-F_1 plants genotyped with KASP	66	X	X	42
BC_3-F_1 plants selected and selfed	8	X	X	12
% recovery recurrent parent (max.)	95.1%			95.2%
BC_3-F_2 plants genotyped with KASP	76	X	X	90
BC_3-F_2 plants selected and selfed	16	X	X	10
% recovery recurrent parent (max.)	98.2%			98.1%

n.d.: not determined
X: work stopped on these crosses

4.2. Hydroponics

i. Standard evaluation system (SES)

Hydroponic assays were performed over the BC_3-LP-F_3 plants, since they had been obtained earlier than BC_3-MS-F_3. In general, a high variation between replicates from the same genotype made it difficult to determinate significant differences and FL478 salt tolerance was lower than the described by Thomson et al. [59].

Each experiment or round of hydroponic assays were independent and tested 18 different genotypes. Even that only 16 plants from the KASP analyses were selected, 3 rounds of hydroponics assays were performed, and a total of 54 plants were tested since in preliminary tests a high variability was observed. This variability was always lower in control trays and was not observed in control trays from the second round.

Figure 25. Over-accumulation of salt in susceptible plants. Crystallized salt can be seen in the shoot because of a lack of ion homeostasis.

Just two days after the salinity treatment was initiated, some plants already showed an over-accumulation of salt in the shoot as seen in Figure 25. This fact is a confirmation that the salt treatment is high enough, because susceptible plants have their ion homeostasis broken rapidly and salt just accumulates in shoot. Lakra et al. [136] observed leaf burning, chlorosis and stunted growth within 48h of exposure to salt stress in the salt sensitive IR64 genotype, under similar hydroponically conditions, but at 200 mM NaCl.

In the first hydroponics round, a variation can be observed in the control trays (Figure 26), where all plants should grow healthy, with some replicates with a SES score over 5 (moderately tolerant, growth severely retarded, most old leaves severely injured). One explication to that variation could be a different vigour between plants. The different genotypes had not achieved a 99.9% return to recurrent parent, consequently they could probably have some genomic regions in heterozygosis segregating. This variability could give more vigour to some replicates. In the third experiment, even one of the salt tolerant lines (FL478-3) scored over 5 in the SES in some replicates in the control trays, making difficult to determinate later salt tolerant genotypes among the plants assayed.

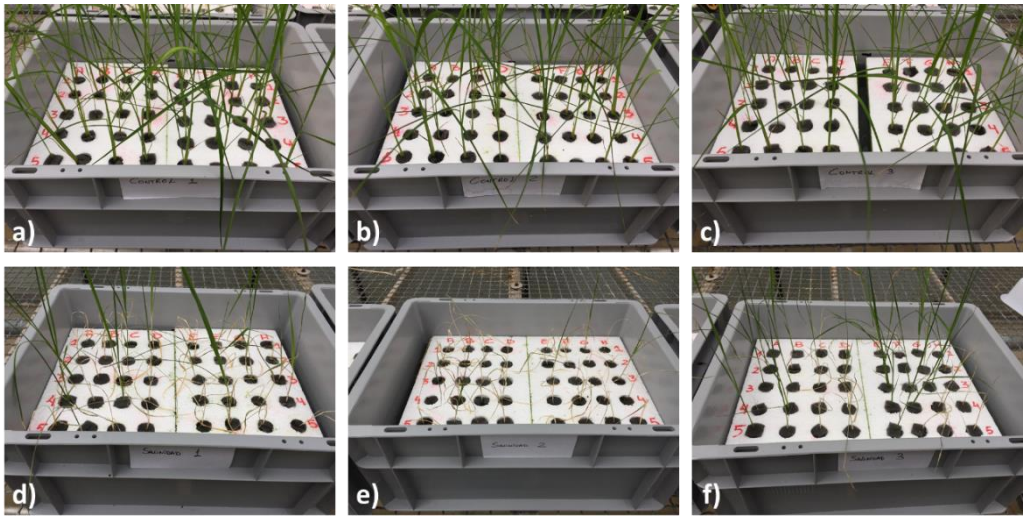

Figure 26. Image *O. sativa* plants from the first hydroponic experiment, at 14 days of 80 mM NaCl treatment. The images a to c show control trays while the images d to f show salinity treatment trays.

Regarding the 80 mM NaCl treatments, the variability between replicates was even higher than the observed in control trays, meaning that two of the replicates were dead (scoring a 9 in the SES) but the other three scored 3 or 1 in the SES (highly tolerant or tolerant) for example for LP-3, LP-15 or LP-17 (Figure 27). Again, this variability could be given only by the difference in vigour, because all replicates were homozygous for

the *Saltol* QTL. However, under this variability could be some other unconsidered salt tolerance QTL present in FL478 [53,55]. In his characterization of the *Saltol* QTL, Thomson already described different alleles of the QTL present in Pokkali (one of the parental of FL478) and the unknown relations between this QTL and other described salt tolerance QTLs [56]. The research and possible identification of these new salt tolerance QTL will be carried out in CRAG with the next hydroponic assays with BC_3-LP-F_4 lines.

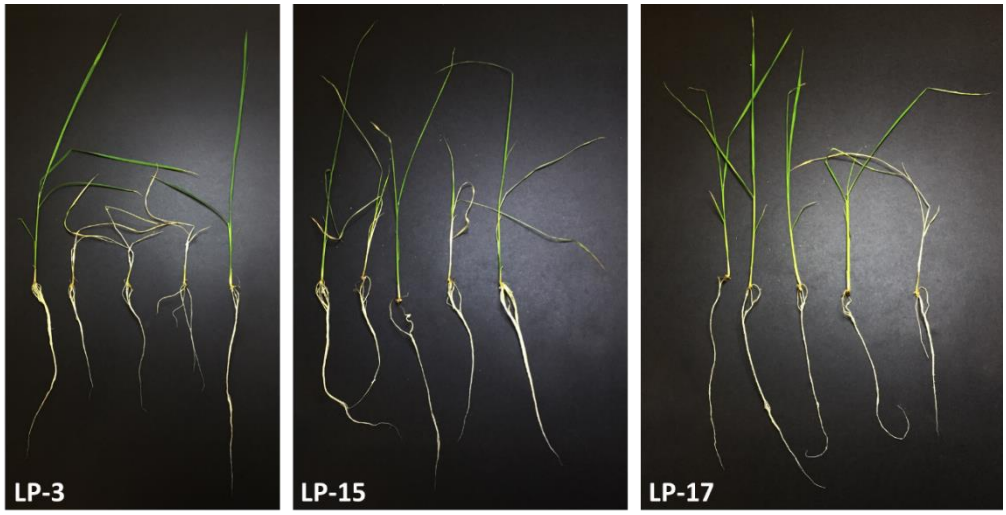

Figure 27. Image of the five replicates of each line (LP-3, LP-15 and LP-17) after the 14 days of NaCl treatment at 80 mM. Some plants are green and healthy, scoring a 1 or 3 in the SES, but other replicates are completely dead and dry (scoring 9 in the SES).

Using the Standardized SES formula (Standardized SES = Salinity SES – Control SES) in Figure 28, those lines that performed similar or even better than FL478 under 80mM NaCl treatment were detected (which scored around 5 as mean of all the replicates). Thus, we can conclude that LP-17 is the most salt tolerant line, even more than FL478, although we must also consider LP-15, LP-19 and LP-31 as salt tolerant lines since they scored similar to FL478.

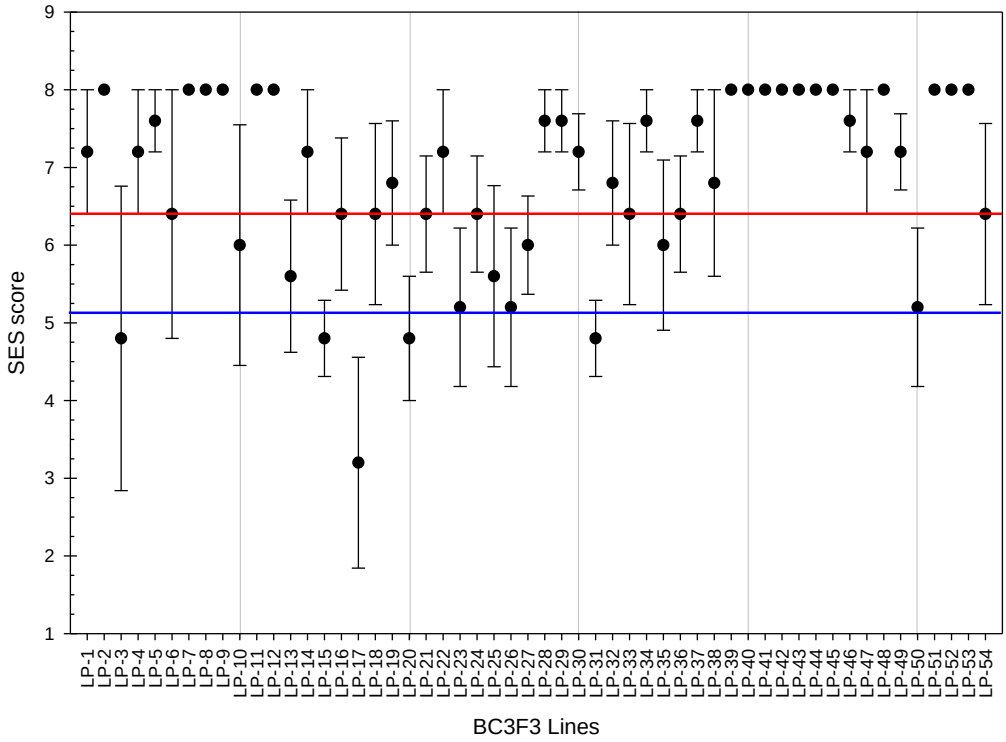

Figure 28. Mean standardized SES score (Standardized SES = Salinity SES – Control SES) for each BC3F3 line (n=5). Blue line represents FL478 mean standardized SES score (n=15), while red line represents PL12 (n=15) mean standardized SES score.

Even that the SES is widely used in salinity hydroponic assays [57,59,135–137], it is a descriptive parameter. The categories that describes are given just by an observation, there is no measurement or analysis. This fact makes the SES a good method for rough discrimination, although more parameters and analyses are needed to determine more accurately any stress tolerance. Some of these parameters can be stress related parameters such as chlorophyll content, malondialdehyde (MDA) accumulation, glutathione (GSH) and hydrogen peroxide (H_2O_2) levels, root-shoot ratio and, especially for salinity stress, Na^+ and K^+ content and ratio [138].

During the course of this thesis, much of these parameters could not be analyzed. Moreover, these experiments are planned to be repeated in the future with descendants from these lines ($BC_3LP\text{-}F_4$) since variation due to unfixed introgressions

will be reduced. Thus, for the $BC_3LP\text{-}F_3$ lines tested only relative chlorophyll content (RCC), fresh weight and length were determined, all the other stress related parameters will be determined over their $BC_3\text{-}F_4$ descendant lines.

ii. Relative chlorophyll content (RCC)

RCC is a parameter frequently used to determinate nitrogen deficiency in plants. However it can also be used to determinate when a plant is stressed for other factors, because the degradation of chlorophyll is a common symptom in most of severe stresses [139,140].

The SPAD 502 Chlorophyll Meter (Konica Minolta, Tokyo, Japan) is a hand spectrometer that allows rapid and precise readings in field with a non-destructive method. It was designed for maize and it is commonly used in fields to determinate the nitrogen fertilization. However, it can be used in other crops and plants, but the utility and consistency of the readings is determined by the leaf structure. In most of cereals measures are consistent, but in di-cotyledons are less reliably [141,142].

In rice, RCC in healthy fully developed plants is usually at a value of 30 – 40 [141], but in plantlets can be a bit lower. In our assays, we considered that below 20 the plant is affected by the salt stress. Variability was higher in NaCl treatment trays than in control trays and hindered the analysis. The reason could be again that some replicates from the same genotype died or were severely affected, while others looked healthy (LP-14, LP-18 and LP-54 in Figure 29). Moreover, some genotypes couldn't be measured since all replicates died. This is remarkable in the third experiment, were almost all genotypes died by salinity.

If we focus in those lines such as LP-3, LP-15 or LP-17, which scored salt-tolerance in the SES, we can see a low RCC in almost all cases, although LP-17 scoring similar data in control and salinity treatment (SPAD values of 27 ± 2 in control tray and 25 ± 5 in NaCl treatment).

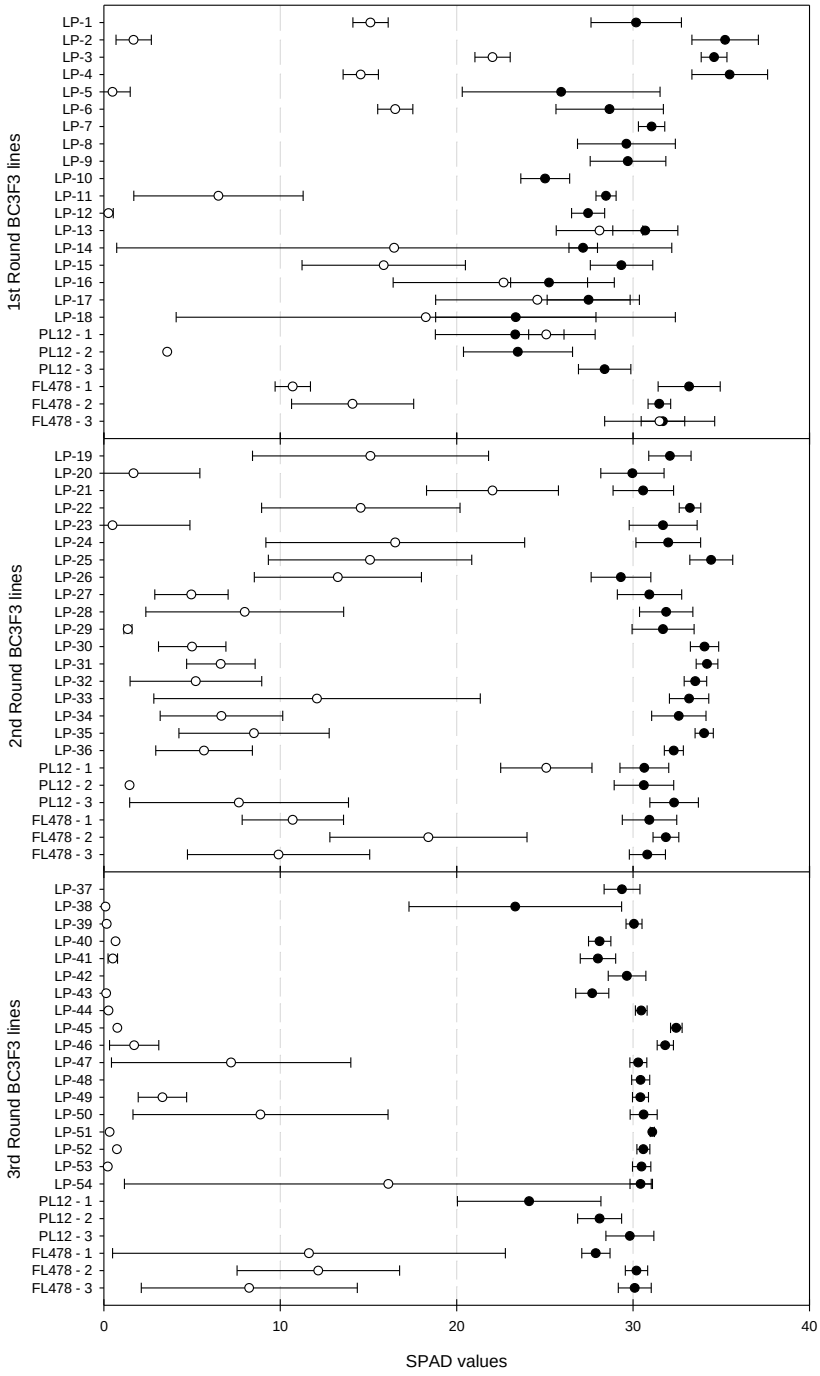

Figure 29. SPAD values for the three rounds of hydroponics. Black dots represent averaged SPAD values for control treatment and White dots for Salinity treatment (80 $dS \cdot m^{-2}$). Significant differences were observed in the three rounds for the treatment factor (p-val < 0.05. In the second round BC3F3 lines, significant differences were observed for lines (p-val < 0.05), but only between PL12 and FL478.

iii. Fresh weight and plant length

Regarding the fresh weight (FW) and plant length, we summarized the results in four graphics that show shoot and root reduction percentage Figure 30. Again, this factor allows us to determine those genotypes that performed similar or better growth than the salt-tolerant parental.

FW reduction in shoot reached 78 ± 1% as mean of all lines. Even that no significant differences were found with the Chi-square test, LP-17 scored definitively a lower FW reduction in shoot (47%). As commented before, salinity stress causes stomatal closure, inhibition of cell elongation, inhibition of photosynthesis and protein synthesis, an overall growth reduction and finally dehydration and leaves senescence [33,35,41]. These symptoms can be also observed in FL478 which, even being a salt tolerant line, scored a 79% of FW reduction in shoot. On the other hand, FW reduction in roots was 51 ± 2% as mean of all lines and significant differences were found ($p\text{-val}<0.05$). In this case, LP-17 showed just a 21% of FW reduction in roots, while FL478 reached a 53%.

For length reduction in shoot and root, results are similar to those observed for FW. Shoot length is more affected by salinity than root, accounting a 43 ± 1% as mean shoot length reduction for all lines and a 20 ± 1% as mean root length reduction, and again significant differences were only found in root length reduction. In one case, LP-17, roots were larger under salinity stress than in non-salinized control (represented as 0% of root reduction). This fact has been explained as a tolerance mechanism: rice plants boost root growth to escape the salinity affected zone and reach no salinized water, as well as reinforce the Casparian bands to reduce the water (and salt) influx [33,143].

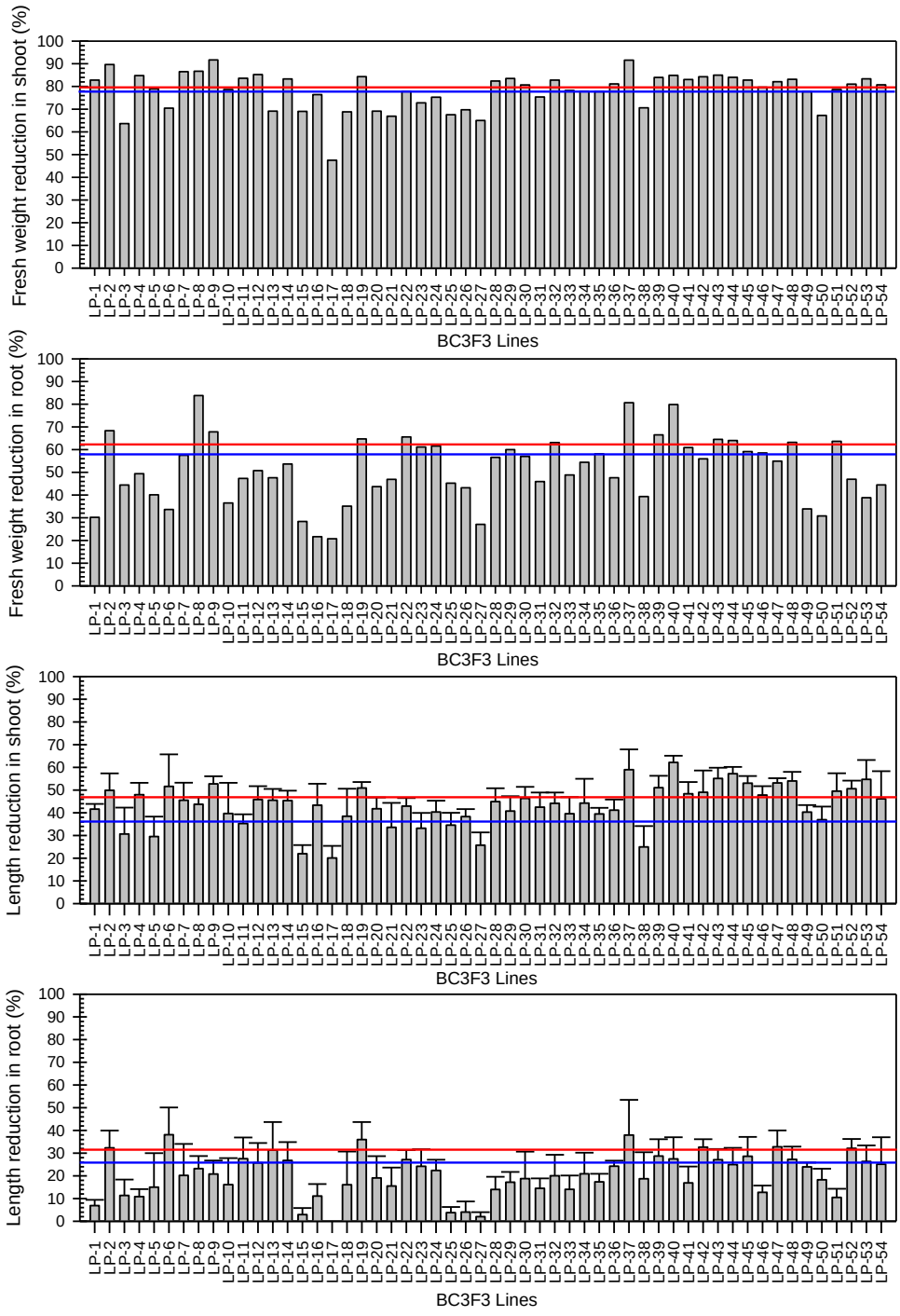

Figure 30. Fresh weight and length reduction (in %) after 14 days of NaCl treatment (at 80 mM) respect control treatment (n=5). Red lines represent mean value for PL12 replicates (n=15) while blue lines represent mean value for FL478 (n=15). Significant differences (p-val < 0.05) were observed for FW reduction in root and Length reduction in root in the Chi-square test.

More replicates of the same experiment must be performed to be able to test such variable lines. Furthermore, as mentioned before, it should be convenient to repeat the assays using KASP selected seeds from one more generation, in order to reduce the variability. Finally, since the salt tolerance in hydroponic assays are not straight correlated to the salt tolerance in field conditions, the most lines will be tested in field even having moderate SES score. The salt tolerance in field conditions of the obtained lines will be verified in two consecutive years, 2018 and 2019 and for the first year, all 54 lines will be assayed. The collaboration with the rice cooperative Càmara Arrossera del Montsià, will guarantee the registration and commercialization of the most salt tolerant lines to improve yield in salinized fields and to fight the apple snail.

4.3. Field assays for Rice Blast resistance

As mentioned before, IR64 is an elite variety from the Philippines that was used as a recurrent parent to obtain IR64-Saltol. IR64, is also a good source of rice blast tolerance genes, as described by Jia et al. [145], so we expected to be able to introgress that blast resistance to our LS and MS lines, along with the salt tolerance. M202-Katy21 is a descendant from Katy line, another blast tolerant line described by Jia et al. Both line share a large fragment in the chromosome 12 that is highly conserved. These field assays expected to find that blast tolerance in the Ebro River Delta, exposing these lines to the *P. oryzae* local strains.

In 2016 field assay some lines from U.S.A and South – East Asia described as rice blast resistant in their origin regions were tested in order to check their blast resistance in Ebro River Delta conditions. Additionally, some hybrids generated the previous year were also tested with the aim of incorporating blast resistance to the local varieties.

One of the first observations was the bad adaptation of some lines such as Bluebelle and M202-Katy21 to the Ebro Delta climate conditions (see Figure 31 and Table 9). These lines had a premature flowering of some panicles before the end of tillering

phase (end of July), which aborted. Another observation was the different growth habitat that have the line 303012, which grew in a very open shape, causing at the end of the season to lose some panicles that fell to the water. On the other hand, 303013 performed really well, had a good adaptation, a vertical growth habitat and a synchronization of nearly all the plant at anthesis, reaching a homogenous grain maturation and a good production (data not shown). Finally, the line IR64-Saltol hadn't even flowered was due to a photoperiod sensitivity, typical for the *indica* lines resulting in no seed production at all.

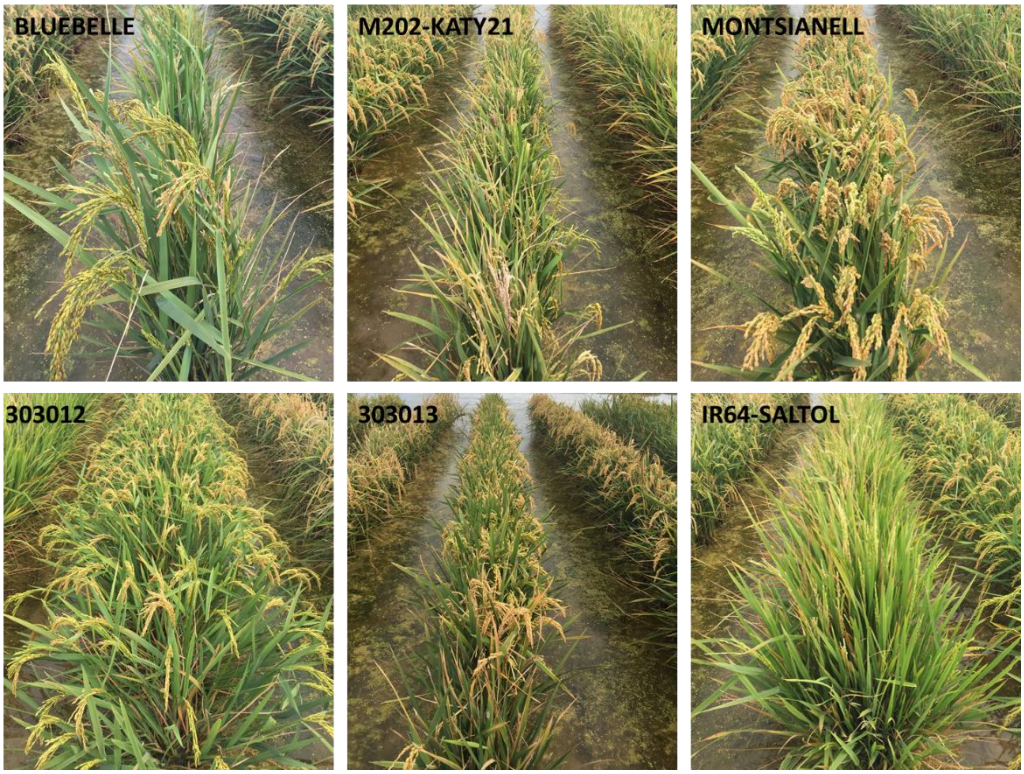

Figure 31. Rice plants (*O. sativa*) tested in the Camara fields. These images, that were taken the first week of September 2016, show the bad adaptation of the lines Bluebelle and M202-Katy21 compared to Montsianell, and 303013, the open shape growth-habit in 303012 and the absence of seeds or mature panicles in IR64-Saltol.

However, the biggest issue was the affectation by *Chilo suppressalis* striped rice stem borer (Figure 32). This Asiatic moth is a serious pest for rice, but ultimately controlled with pesticides [146,147]. Moreover, local rice varieties have adapted to them with some basal resistance characteristics like reinforced cell walls, silica spicules or increase in trichomes (personal communication from Càmara Arrossera del Montsià). This widespread moth, that habits in East Asia, India and Indonesia, but has reached Japan, Australia, Hawaii and Europe, has not arrived yet to U.S.A. [148]. Therefore, some lines from California (Bluebelle, M202-Katy21 and some of their hybrids) suffered heavily this pest attacks. At the end of the season all plants were so destroyed that it wasn't possible to evaluate the plants for rice blast resistance.

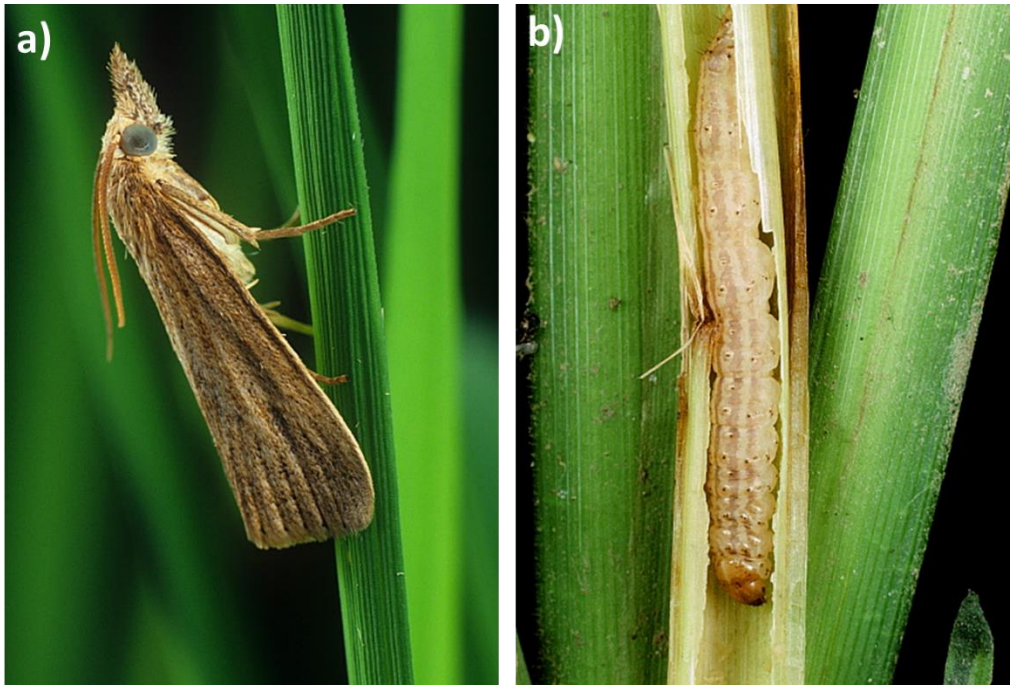

Figure 32. Rice stem borer (*Chilo suppressalis*). a) this lepidopteran lays the eggs in rice stems and then b) the larvae perforates the stem and feeds from the internal rice tissues. This pest usually causes death of infected plants, but damages can be reduced with insecticide treatments and pheromones traps. Images belong to Nigel Cattlin (Berkshire, UK).

Surprisingly, the 3030XX lines resisted the stem borer somehow and were evaluated for rice blast resistance. However, 2016 was a bad year for fungus infections, high temperatures and low humidity stopped the rice blast spread. That fact made the native variety, Montsianell, usually susceptible to rice blast, to perform and develop without almost any symptom.

Other authors used controlled conditions (mostly greenhouse conditions) to perform inoculation of certain *P. oryzae* isolates or strains, usually to test or discover particular resistance genes [93,94,149,150] . However, much less work concerning fungus susceptibility field assays are published, where plenty of *P. oryzae* strains are usually present in addition to other fungus and pathogens.

After that field assay we could not advance in blast resistance studies or adopt a more molecular approach, so we selected individual plants from the best lines and repeated the assay in 2017 including the F3 generation of those hybrids. Furthermore, we included Olesa as one of the native varieties and removed IR64-Saltol and 303011 from the assay.

The stem borer attacks went even worst during the 2017 field assay (see Figure 33 and Table 10). Bluebelle and M202-Katy21 were totally damaged a month before the last evaluation. Moreover, we observed a call effect of Bluebelle, since all the contiguous lines appeared heavily affected by stem borer, even native varieties, which show some resistance to this pest.

Luckily, the climatic conditions on 2017 allowed a higher fungus virulence and some symptoms could be evaluated in the tested lines. Nonetheless, the tested parental lines did not demonstrate a higher resistance to rice blast than Olesa or Montsianell. Only two hybrids, H5-F3-12xBB and H1-F3-MNTxM202 seemed to be resistant although some individuals were attacked by *Fusarium sp.*, another fungus that affects rice. This could be a random attack although a susceptibility to that fungus is possible, so more observations are needed. From both hybrid lines, some plants were selected to be tested in the near future.

Figure 33. Rice plants (*O. sativa*) tested in the Camara fields. The first image was taken the last week of September 2016, and shows the destruction caused by the rice stem borer (*C. suppressalis*) in M202-Katy21 and Bluebelle lines, compared to Montsianell and 303013. The other two images show a detail of these stem borer susceptible lines the last week of September 2017.

The hybrid H5-F3-12xBB was not only blast resistant, but also stem borer resistant, showing a homogeneity in cycle and height, despite being an F3 generation. The other four hybrid lines from the cross 12xBB were surprisingly identical between them even being from different F2 plants. Unfortunately, they were not agronomically acceptable since they had low interest characters such as small panicles and thin stems.

Regarding the 11xMNT hybrids, some individuals were selected from the H1-F3-11xMNT hybrid line, because of their low height, about 70 cm, and big and large panicles, although presenting undesired traits such as long awns that must be eliminated in further generations.

Table 9. Field evaluation of the blast tolerant candidate rice lines and some F2 hybrids with Mediterranean rice varieties last week of September 2016. Height is the mean ± std. error for 3 random plants. Stem borer refers to *Chilo suppressalis* affectation. Rice blast refers to *Pyricularia oryzae* affectation. Some lines were so destroyed by C*. suppressalis* that blast affectation was not determined (n.d.).
*Cycle: following Lancashire et al. uniform code [129].
**Unable to be determined that day. Valor from the last evaluation (3 weeks before)

LINES	Homogeinity	Tillering	Height (cm)	Cycle*		Stem borer	Rice blast	OBSERVATIONS	NUMBER OF PLANTS SELECTED
MONTSIANELL	+ + +	+ + +	93 ± 2	71	79	+	+ + +	Native variety.	-
BLUEBELLE	+ + +	+ + +	98 ± 2	50**		-	n.d.	U.S.A. variety, not well adapted. Susceptible to Xilo suppressalis.	-
M202-KATY21	+ + +	+ + +	100	75	83	-	n.d.	U.S.A. variety, not well adapted. Susceptible to C. suppressalis.	-
IR64 SALTOL	+ + +	+ + +	88 ± 2	29		+ + +	+ + +	Philippine variety. Photoperiod afffected: heavy delay in flowering	-
303011	+ + +	+ +	88 ± 2	69		+	+ + +	U.S.A. variety, adapted. Adjusted cycle and long panicle.	-
303012	+ + +	+ +	72 ± 2	69		+ + +	+ + +	U.S.A. variety, adapted. Really wide growing habit. Thick shoots and glabrous.	-
303013	+ + +	+ +	85	69		+ + +	+ + +	U.S.A. variety, well adapted.Full plant anthesis sincronitzation.	-
H1-F2-OLSxBB	-	+ +	92 ± 12	41	69	-	+ +	Some plants without C. suppressalis. High variability.	1
H1-F2-BBxOLS	-	+ +	103 ± 10	43	85	-	+ +	Some plants without C. suppressalis. High variability.	1
H1-F2-12xBB	+	+ + +	80 ± 3	69		+ + +	+ + +	Diverse grow habits (some open some more vertical). Homogeinity in cycle.	5
H1-F2-BBx13	-	+ + +	103 ± 15	41	77	-	n.d.	High susceptibility to C. suppressalis.	1
H1-F2-M202xMNT	-	+ + +	87 ± 9	75	87	-	n.d.	High susceptibility to C. suppressalis. High variability.	0
H1-F2-MNTxM202	-	+ + +	97 ± 12	80	87	-	n.d.	High susceptibility to C. suppressalis. High variability.	1
H1-F2-11xMNT	-	+ + +	98 ± 15	39	87	+	+ + +	Some plants without C. suppressalis. High variability. Some plants with large awns.	6

LEGEND				
Code	**Homogeinity**	**Tillering**	**Chilo**	**Rice blast**
+ + +	>90% homogenous	>40 tillers	0-1affected plants	0 - 0.5% leaf affectation
+ +	50 - 75% homogenous	20-40 tillers	2-5 affected plants	0,5 - 1% leaf affectation
+	25 - 50 % homogenous	10-20 tillers	5-10 affected plants	1 - 2% leaf affectation
-	<25% homogenous	<10 tillers	>10 affected plants	>2% leaf affectation

Table 10. Field evaluation of the blast tolerant candidate rice lines and some F3 hybrids with Mediterranean rice varieties last week of September 2017. Height is the mean ± std. error for 3 random plants. Stem borer refers to *Chilo suppressalis* affectation. Rice blast refers to *Pyricularia oryzae* affectation. Some lines were so destroyed by C*. suppressalis* that blast affectation was not determined (n.d.).
*Cycle: following Lancashire et al. uniform code [129].
**Unable to be determined that day. Valor from the last evaluation (3 weeks before)

LINES	Homogeinity	Tillering	Height (cm)	Cycle*		Stem borer	Rice blast	OBSERVATIONS	NUMBER OF PLANTS SELECTED
OLESA	+ + +	+	77 ± 2	89		-	+ + +	Native variety. Affected by C. suppressalis even not being susceptible in field.	-
MONTSIANELL	+ + +	+ +	82 ± 3	87		+	+ +	Native variety.	-
BLUEBELLE	+ + +	+	73 ± 2	39**		-	n.d.	U.S.A. variety, not well adapted. Totally destroyed by C. suppressalis.	-
M202-KATY21	+ + +	+ +	82 ± 2	69**		-	n.d.	U.S.A. variety, not well adapted. Totally destroyed by C. suppressalis.	-
303012	+ + +	+ +	72 ± 2	77		-	+ +	U.S.A. variety, adapted. Really wide growing habit. Thick shoots and glabrous.	-
303013	+ + +	+ +	73 ± 2	77	85	-	+ +	U.S.A. variety, well adapted. All plants have some tiller affecte by C. supressalis.	-
H1-F3-OLSxBB	+	+ +	83 ± 4	85	87	-	+	Too tall. High variability. High fungus susceptibility. High dehiscence in panicle.	1
H1-F3-BBxOLS	+	+ +	82 ± 7	87		-	+	Too tall. High variability. High fungus susceptibility.	0
H1-F3-12xBB	+ + +	+ +	72 ± 2	77	85	+	+ +	Almost all hybrids 12xBB are nearly identical. Small panicles and thin shoots. Low fungus affectation.	0
H2-F3-12xBB	+ +	+ +	70 ± 3	85		-	+		
H3-F3-12xBB	+ + +	+ +	68 ± 2	85		+	+ +		
H4-F3-12xBB	+ + +	+ +	68 ± 2	85		+	+ +		
H5-F3-12xBB	+ + +	+ +	68 ± 3	87		+ + +	+ + +	Highly homogeinity, resistant to C. suppresalis and fungus. Thick leaves.	4
H1-F3-BBx13	-	+ +	73 ± 6	77	85	-	+	Large panicles. Some plants highly affected by P. oryzae. Some sterility.	1
H1-F3-MNTxM202	+ +	+ +	67 ± 3	85		+ +	+ + +	Some plants ffected by Fussarium sp.	2
H1-F3-11xMNT	-	+	77 ± 7	85		+	+ +	Some plants have large awns. Reduced height and large panicles.	4
H2-F3-11xMNT	+	+ +	78 ± 6	77	85	+ +	+	Large panicles. Some plants highly affected by P. oryzae.	0
H3-F3-11xMNT	+	+	87 ± 7	77	85	+	+	Some plants have large awns. High variability. High affectation of P. oryzae.	0
H4-F3-11xMNT	-	+ +	85 ± 12	85	87	+	+ +	Some plants have large awns. Some plants have large panicles.	0
H5-F3-11xMNT	-	+ +	85 ± 13	85	87	-	+	Some plants have large awns. Susceptibility to fungus.	0
H6-F3-11xMNT	+	+	92 ± 6	77	85	-	+	Too tall. Some plants have large awns. High variability.	0

CONCLUSIONS

5. CONCLUSIONS

1. We have successfully introgressed *Saltol* QTL in Mediterranean *japonica* rice varieties from *indica* Asiatic rice lines. The use of the MABC, combined with embryo rescue, has allowed us to make the transfer of genotype in less than 3 years, and reach a BC_3F_3 with more than 98% of return to recurrent parent.

2. Embryo rescue is a useful biotechnological tool to speed up breeding process. The use of this technique in immature seeds of 10 days after pollination makes possible to skip 30 days of rice life cycle.

3. The salt tolerance of the BC_3F_3 lines from the PL12xFL478 cross, tested in hydroponic assays, was not the expected. One line, LP-17, was more salt tolerant than FL478 and only three lines proved a salt tolerance similar to FL478, according to the SES evaluation. However great variability was observed between replicates of each line, including parental lines, which means homozygote BC_3F_4 lines must be tested in the future.

4. The relative chlorophyll content (RCC) was not a determinant parameter to evaluate salt tolerance and a high variability between replicates was observed again. Some degree of heterozygosity could be giving these lines a variation in vigour between replicates or a variation in other unconsidered salt tolerance QTL that are present in FL478.

5. Fresh weight (FW) and plant length of the salt tolerance tested lines showed a similar reduction, being higher in the shoot than the root in both cases. This evidence is well described as a tolerance method under salt stress, the plant reduces shoot growth and boosts root growth to find a salt free region in the soil.

6. Finally, field assays for rice blast (*Pyricularia oryzae*) resistance of a panel of foreign and local rice lines were severely affected by the stem borer pest (Ch*ilo suppressalis*). Some of these lines, which are from U.S.A., are not adapted to this pest, so they were totally destroyed both years and no prominent line was selected. For future assays, more stem borer pest control measures must be taken and new lines must be assayed to identify some blast resistance phenotype.

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ANNEXES

7. ANNEXES

7.1. Statistics

Two ways ANOVA for SPAD values - Experiment 1

Análisis de Varianza para 1- SPAD value - Suma de Cuadrados Tipo III

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
EFECTOS PRINCIPALES					
A:1-Treatment	4488,64	1	4488,64	44,15	**0,0000**
B:1- Line	633,96	7	90,5657	0,89	0,5201
RESIDUOS	5591,2	55	101,658		
TOTAL (CORREGIDO)	10567,6	63			

Todas las razones-F se basan en el cuadrado medio del error residual

Pruebas de Múltiple Rangos para 1- SPAD value por 1-Treatment

Método: 95,0 porcentaje Bonferroni

1-Treatment	*Casos*	*Media LS*	*Sigma LS*	*Grupos Homogéneos*
Salinity	27	14,0612	1,99931	X
Control	37	31,4254	1,66973	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
Control - Salinity	*	17,3642	6,02189

* indica una diferencia significativa.

Pruebas de Normalidad para RESIDUOS

Prueba	*Estadístico*	*Valor-P*
Estadístico W de Shapiro-Wilk	0,977997	0,572298

Two ways ANOVA for SPAD values - Experiment 2

Análisis de Varianza para 2- SPAD value - Suma de Cuadrados Tipo III

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
EFECTOS PRINCIPALES					
A:2- Treatment	2629,58	1	2629,58	150,69	**0,0000**
B:2- Line	426,754	7	60,9649	3,49	**0,0056**
RESIDUOS	645,67	37	17,4505		
TOTAL (CORREGIDO)	4105,32	45			

Todas las razones-F se basan en el cuadrado medio del error residual

Pruebas de Múltiple Rangos para 2- SPAD value por 2- Treatment

Método: 95,0 porcentaje Bonferroni

2- Treatment	*Casos*	*Media LS*	*Sigma LS*	*Grupos Homogéneos*
Salinity	9	7,06467	1,59037	X
Control	37	28,3036	0,695043	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
Control - Salinity	*	21,2389	4,04228

* indica una diferencia significativa.

Pruebas de Múltiple Rangos para 2- SPAD value por 2- Line

Método: 95,0 porcentaje Bonferroni

2- Line	*Casos*	*Media LS*	*Sigma LS*	*Grupos Homogéneos*
PL12-2	4	13,1903	2,13301	X
10	4	14,4055	2,26076	XX
12	7	15,1345	1,62185	XX
11	7	17,6345	1,62185	XX
8	5	19,0005	2,05876	XX
9	5	19,1005	2,05876	XX
7	5	20,4205	2,05876	XX
FL478-2	9	22,5867	1,39577	X

Pruebas de Normalidad para RESIDUOS2

Prueba	*Estadístico*	*Valor-P*
Estadístico W de Shapiro-Wilk	0,966002	0,303475

Two ways ANOVA for SPAD values - Experiment 3

Análisis de Varianza para 3- SPAD value - Suma de Cuadrados Tipo III

Fuente	*Suma de Cuadrados*	*Gl*	*Cuadrado Medio*	*Razón-F*	*Valor-P*
EFECTOS PRINCIPALES					
A:3- Treatment	402,55	1	402,55	6,36	**0,0149**
B:3- Line	658,091	7	94,0129	1,48	0,1939
RESIDUOS	3230,29	51	63,339		
TOTAL (CORREGIDO)	4292,27	59			

Todas las razones-F se basan en el cuadrado medio del error residual

Pruebas de Múltiple Rangos para 3- SPAD value por 3- Treatment

Método: 95,0 porcentaje Bonferroni

3- Treatment	*Casos*	*Media LS*	*Sigma LS*	*Grupos Homogéneos*
Salinity	22	22,3371	1,82694	X
Control	38	27,9543	1,29534	X

Contraste	*Sig.*	*Diferencia*	*+/- Límites*
Control - Salinity	*	5,6172	5,14596

* indica una diferencia significativa.

Pruebas de Normalidad para RESIDUOS3

Prueba	*Estadístico*	*Valor-P*
Estadístico W de Shapiro-Wilk	0,944769	0,161091

Chi-square Test for Shoot Weight reduction (vs. FL478)

Prueba	*Estadístico*	*Gl*	*Valor-P*
Chi-Cuadrada	24,453	54	0,9998

Chi-square Test for Root Weight reduction (vs. FL478)

Prueba	*Estadístico*	*Gl*	*Valor-P*
Chi-Cuadrada	116,509	54	**0,0000**

Chi-square Test for Shoot Length reduction (vs. FL478)

Prueba	*Estadístico*	*Gl*	*Valor-P*
Chi-Cuadrada	55,529	54	0,4169

Chi-square Test for Root Length reduction (vs. FL478)

Prueba	*Estadístico*	*Gl*	*Valor-P*
Chi-Cuadrada	154,176	54	**0,0000**