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Pollen embryogenesis for rice plant production from selected genotypes cultivated in the Mondego river valley

Dissertação apresentada à Universidade de Coimbra para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biodiversidade e Biotecnologia Vegetal, realizada sob a orientação científica do Professor Doutor Jorge Canhoto (Universidade de Coimbra).

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Atm – Atmosphere

2,4-D – Dichlorophenoxyacetic acid

BAP – 6-Benzylaminopurine

DI – DNA index

HSPs – Heat-shock proteins

IAA – Indole-3-acetic acid

NAA – α-naphtaleneacetic acid

PGRs – Plant growth regulators

PI – Propidium iodide

SE – Standard error

Resumo

O arroz (*Oryza sativa* L.) é atualmente o alimento mais importante do mundo, sendo a base da alimentação de mais de metade da população mundial. Na Europa, Portugal é o principal consumidor, com os vales dos rios a serem as regiões privilegiadas para a cultura deste cereal. Ao contrário de outros países produtores, os programas de melhoramento ainda se baseiam muito nos métodos convencionais. A androgénese representa uma ferramenta muito útil para o desenvolvimento de novas variedades de arroz, reduzindo o tempo, o trabalho e os custos na obtenção de objetivos específicos no melhoramento do arroz. A cultura de anteras é a técnica mais aplicada para induzir a androgénese.

Neste trabalho, que teve origem em 2010, a cultura de anteras foi efetuada em diversas linhas experimentais e variedades de arroz produzidas no campo experimental do Bico da Barca no vale do rio Mondego. As panículas de arroz sofreram um pré-tratamento com frio durante 10 dias a 4ºC em 2012. As anteras foram excisadas das espiguetas e inoculadas em meio N6 modificado com 4 tratamentos de indução diferentes: A1 - 2 mg/L 2,4-D e 3% de sacarose; A2 - 2 mg/L 2,4-D, 1.5 mg/L kinetina e 3% de sacarose; A3 - 1 mg/L 2,4-D, 1mg/L NAA, 2 mg/L kinetina e 3% de sacarose; A4 - 2 mg/L 2,4-D e 6% de sacarose. Os calos polínicos formados foram transferidos para 4 meios de regeneração diferentes consistindo todos em meio base MS suplementados com 3% de sacarose: D_3R - BAP (1 mg/L) e NAA (0.1 mg/L); RR1 - kinetina (4 mg/L) e NAA (1 mg/L); RR2 - kinetina (2 mg/L) e NAA (0,5 mg/L); RR3 - kinetina (2 mg/L) e NAA (1 mg/L).

As plântulas regeneradas foram aclimatizadas numa câmara de crescimento em condições controladas e num substrato muito semelhante àquele encontrado nos campos de cultura de arroz, sendo depois analisadas através de citometria de fluxo. Os resultados mostraram, no geral, que o genótipo e as condições de crescimento das plantas dadores desempenham um papel importante na indução de calos e na regeneração de plantas, com algumas linhas a apresentarem taxas de indução de calos e regeneração de plantas maiores que outras linhas cultivadas nos mesmos meios. O meio A3 deu a melhor frequência de indução de calos e o meio RR1 apresenta os

melhores resultados na regeneração de plantas. A produção de plantas albinas foi observada, estando relacionada com todos os meios de indução e regeneração.

A análise por citometria de fluxo revelou a obtenção tanto de plantas haplóides como de diplóides, com a presença também de alguns indivíduos triplóides, tendo sido os haplóides obtidos em maior percentagem. As plantas diplóides podem ter duas origens diferentes: através da duplicação espontânea dos cromossomas ou da formação de calos dos tecidos somáticos das anteras. As plantas resultantes da duplicação espontânea de cromossomas são chamadas de dihaplóides e são as mais importantes para os programas de melhoramento. As plantas obtidas estão ainda a ser analisadas e poderão ser usadas em programas de melhoramento com o objetivo de melhorar o sucesso dos métodos para a produção de novos cultivares.

Palavras-chave: androgénese, arroz, citometria de fluxo, cultura de anteras, dihaplóides

Abstract

Rice (*Oryza sativa* L.) is currently the main food crop in the world, feeding more than half the global population. Portugal is the main consumer of rice in Europe, with river valleys as privileged regions to exploit the culture of this crop. In opposition to other producer countries, breeding programs still rely mainly on conventional methods. Androgenesis represents a powerful tool for the development of new varieties of rice, reducing time, labor and money to achieve specific goals in rice breeding. Anther culture is the most common technique used to induce androgenesis.

In the present work, which started in 2010, anther cultures where initiated from different experimental lines and cultivars of rice produced in the Bico da Barca experimental field in the Mondego river valley. Rice panicles were cold-pretreated for 10 days at 4ºC on 2012. The anthers were excised from spikelets and cultured in modified N6 medium with four different induction treatments: A1 - 2 mg/L 2,4-D and 3% sucrose; A2 - 2 mg/L 2,4-D, 1.5 mg/L kinetin and 3% sucrose; A3 - 1 mg/L 2,4-D, 1mg/L NAA, 2 mg/L kinetin and 3% sucrose; A4 - 2 mg/L 2,4-D and 6% sucrose. Pollen calli, were transferred to four different regeneration media consisting in MS basal medium supplemented with 3% sucrose and: D_3R - BAP (1 mg/L) and NAA (0.1 mg/L); RR1 - kinetin (4 mg/L) and NAA (1 mg/L); RR2 - kinetin (2 mg/L) and NAA (0.5 mg/L); RR3 - kinetin (2 mg/L) and NAA (1 mg/L).

The regenerated plantlets were acclimatized in controlled conditions in a growth chamber and in soil collected at Bioc da Barca, being analyzed afterwards by flow cytometry. The results showed, in general, that genotype and growth conditions of donor plants, play an important role in the induction of callus and plant regeneration, with some lines revealing a higher rate of callus formation and plant regeneration than other lines cultured in the same media. A3 medium gave best induction frequency of pollen calli and RR1 showed the best results for plant regeneration. The production of albino plants was observed and related to all induction and regeneration media.

Flow cytometry analyses revealed the presence of both haploid and diploid plants among the regenerants. A few triploids were also found. The diploid plants may have two different origins: through spontaneous chromosome

doubling or callus formation from anther somatic tissues. The plants resulting from spontaneous chromosome doubling are called dihaploids and are the ones with great interest for breeding programs. The plants so far obtained are still being analyzed and could be used for breeding programs with the aim to improve the yield of the methods for new varieties development.

Key words: androgenesis, anther culture, dihaploids, flow cytometry, rice

1. Introduction

Introduction

1.1. Biotechnology – methodologies and applications

According to recent estimations (FAO, 2012), world agriculture will need to feed more than 9 billion people by 2050, about 2 billion more than today. Most of the population growth will occur in countries where hunger and natural resource degradation are already prevailing. To feed this larger mostly urban population, food production will have to increase by 70%; for example, cereal production will need to reach about 3 billion tons, compared with the actual 2.1 billion (Altman and Hasegawa, 2012). It should be also mentioned that the problem of food supply refers not only to caloric demands, but also to increased intake of vitamins, essential minerals, and other important nutritional factors via production of biofortified food to improve the health of undernourished people, especially in children and in poor countries.

Three major causes can be mentioned in the urgent need for alternative biotechnologies, mostly in plants: 1) the already referred increase of world population; 2) recognition that human health is affected by disease-causing pathogenic organisms and by the nutritional quality of foods; and 3) global climatic changes which may be responsible for adverse biotic and abiotic stresses to crops and ecosystems. It is estimated that in developing countries 80% of the necessary food production increase would come from yield increase and cropping intensity, and only 20% from expansion of arable land, which emphasizes an even greater need for improved agricultural technologies and biotechnologies (Altman and Hasegawa, 2012).

Therefore, in a world where the population growth is overtaking food production, agricultural and plant biotechnologies need to be swiftly implemented. Classical agriculture is no longer sufficient to supply the demand of developed and developing countries worldwide for food production, as well as for new plant commodities; thus, in the last decade a phenomenally greater understanding of plants biology took place, which allowed the increase of plant-derived food, fiber, biopolymer, biofuel, and metabolite production.

In the last years science has experienced a huge breakthrough thanks to diverse new methodologies and a respective range of applications. Such innovations are usually known as Biotechnology, which can be described as the commercial application of all types of biological reactions and processes. Living cells and its components can be used to produce a large number of useful compounds like therapeutics and other products also important (Nair, 2008). On this regard, advances in molecular biology, structural and functional genomics, bioinformatics and related fields have led to the development of biotechnology driven tools, methods, and products, making biotechnology a key area for the 21st century. Focusing only in Plant Biotechnology, this one refers to the manipulation of plants to obtain new characteristics and using them to achieve certain products or performing several functions (Canhoto, 2010).

Inside the extensive field of Plant Biotechnology, plant tissue culture, as it is generically referred to, is the most prominent plant biotechnology tool. It was developed primarily as a platform for basic research on cellular and tissue differentiation, and morphogenesis and hormone identification and function (Altman and Hasegawa, 2012). Plant tissue culture is a technique of culturing plant cells, tissues and organs on synthetic media under aseptic environment and controlled conditions of light, temperature, and humidity (Dagla, 2012), in a way to avoid contamination by microorganisms. The technique depends mainly on the concept of totipotency of plant cells which refers to the ability of a single cell to express the full genome by repeated divisions (Hussain *et al*., 2012). There are many applications for this technique including plant micropropagation, production of secondary metabolites, genetic transformation and somatic breeding methodologies, as protoplast fusion and generation of haploids (Canhoto, 2010).

The beginning of the XX century was marked by systematic efforts made by several researchers to culture excised plant tissues and organs to understand their growth and development under controlled laboratory conditions. In 1838, Schlein and Schwann proposed that cells are the basic structural units of all living organisms, capable of autonomy and therefore it should be possible for each cell to regenerate into a whole plant (Hussain *et al*., 2012). Based in this concept of totypotency, in 1898 the German botanist Gottlieb Haberlandt was the first to culture isolated, fully differentiated cells. His

goal was to achieve continued cell division in explanted tissues on nutrient media. Although he succeeded in maintaining isolated leaf cells alive for extended periods, the cells failed to divide because the lack of plant hormones in the nutrient media (Dagla, 2012). In 1926, Haberlandt's vision came true with the discover of the first plant growth hormone, the indole-3-acetic acid (IAA) by Went: Later on (1939), White, Gautheret and Nobecourt reported unlimited growth of callus cultures (Dagla, 2012; Hussain *et al*., 2012). This discovery boosted the researchers to understand the processes controlling development in plants (Pais, 2003). During the 50's, tissue culture has achieved great successes, and in 1954 Muir was the first to break callus tissues into single cells, and Reinert and Steward in 1959 regenerated embryos from callus clumps and cell suspensions of *Daucus carota* (Hussain *et al*., 2012).

A mass of proliferating non-organized cells is usually known as "callus" (Dagla, 2012), and its formation is frequent in tissue culture. Some callus have the ability to regenerate whole plants in the appropriate medium and conditions. Plant tissue culture media contain all the nutrients required for the normal growth and development of plants such as macronutrients, micronutrients, vitamins, a carbon source, and other organic components, the most important being the plant growth regulators (PGRs). PGRs play an essential role in determining the developmental pathway of plant cells and tissues in culture. Auxins, cytokinins and gibberellins are the most commonly used PGRs (Hussain *et al*., 2012). The type and concentration of hormones used vary according to the explants used and to the objectives of the culture process.

One of the most important applications of plant biotechnology is haploid production. The general term "haploid" is applied to any plant possessing half the chromosome number of the sporophyte. Haploid plant formation may occur spontaneously in nature, resulting from the proliferation of haploid cells of the embryo sac but also, although less often, from the proliferation of male gametes (Bajaj, 1983).

Haploids have attracted great interests of geneticists, plant embryologists, physiologists and breeders that long wished for the production of haploids due to their potential for plant breeding. The most important is that homozygous diploids (pure lines), can be obtained in one generation via chromosome

doubling of the haploid plants, hence drastically reducing the breeding cycle, in comparison to repeated cycles of inbreeding in self-pollinating crops, the traditional method of homozygous production (Maheshwari *et al*., 1980; Dunwell, 1985; Han and Hongyuan, 1986). Haploids may also be used to easily detect mutations and for the recovery of unique recombinants. Haploids possess only one allele at each locus which means that they cannot be hidden by dominant alleles. This is particularly valuable in mutation breeding and research of mutant genetics (Bajaj, 1983; Han and Hongyuan, 1986). Another application is to facilitate genetic analysis of recombinant gametes, since in haploids gametic genotypes are fully expressed at plant level (Han and Hongyuan, 1986). Haploids can also be useful in genetic transformation since if transformation is induced at the haploid level, chromosome doubling will give origin to genetic transformed plants homozygous for the transformed character (Huang, 1992).

For breeding purposes is of great importance, to induce haploids in higher numbers (Reinert and Bajaj, 1977). Haploid formation has been reported in many species of angiosperms belonging quite distinct families and genus. However, for most of these species, haploid based breeding has been quite limited due to the low frequencies of induction (Han and Hongyuan, 1986; Wedzony *et al*., 2009). Haploids can usually be induced by several methods, including ionizing irradiation and radioisotopes, thermal shocks, distant hybridization, delayed pollination, application of abortive pollen, spraying with various chemicals, *in vitro* parthenogenesis, chromosome elimination by culture of young embryos, culture of isolated pollen and protoplasts and the *in vitro* culture of the excised anthers or isolated microspores/pollen (Bajaj, 1983), this last method became the more important and reliable in the last 50 years.

In 1922, Blakeslee, while working with *Datura stramonium*, reported the natural occurrence of haploids. Later on (1953), Tulecke succeeded in growing calli from pollen grains of the gymnosperm *Ginkgo biloba* when cultured *in vitro*. This calli had the haploid number of chromosomes; however it has not been possible to regenerate plants from them (Nitsch, 1977; Bajaj, 1983). Since this pioneer reports, efforts have been made to improve haploid plant production. It was only in 1964 that Guha and Maheshwari (1964, 1966) first reported the direct development of haploid embryos from microspores of *Datura innoxia*

(Solanaceae) by culture of excised anthers *in vitro*, which was later confirmed to have origin in pollen grains (Guha and Maheswari, 1967), given rise to a huge impulse to studies on the potentialities of a number of other angiosperm pollen for cell division and growth *in vitro* in several laboratories all over the world.

After the first discoveries of Guha and Maheshwari (1964) a series of studies were made to obtain the same success in others species. Among dicotyledons, plants belonging to Solanaceae, have consistently proved to be a good source for induction of the pollen embryogenesis. On the other hand, in monocotyledons, there is a long list of plants belonging to Gramineae (Poaceae) in which haploid plants have been obtained, including several grasses and major crop plants, such as rice, wheat and maize (Canhoto, 2010; Olmedilla, 2010). So, it's clear that Gramineae shares with Solanaceae a proeminent role as suitable material for research on androgenesis (Raghavan, 1986). Most solanaceous plants have proved versatile in that embryoids developed directly from the pollen, unlike the monocots group which is characterized by the formation of a callus before organogenesis occurs (Narayanaswamy and George, 1982).

In angiosperms, the micropore is the product of a reduction division of the pollen mother cell and is the basic entity from which the male gametophyte arises (Raghavan, 1986). Although during microgametogenesis pollen grains are programmed for differentiation into gametes and to form pollen tubes, immature pollen or microspores of many plants may also enter an alternative developmental pathway in a relatively simple salt medium, in which they initiate indeterminate growth giving rise to mature plants with the haploid or gametic set of chromosomes, a process usually known as pollen embryogenesis, microspore embryogenesis or androgenesis (Raghavan, 1986; Reynolds, 1997), this last term being used in this work.

Before analysis of the sporophytic pathway of pollen development, it is suitable to briefly review normal pollen ontogeny.

1.2. Microsporogenesis and microgametogenesis

Pollen formation is the consequence of two temporal successive events occurring in the anthers: microsporogenesis and microgametogenesis. The first one begins when a diploid microsporocyte undergoes meiosis to produce a tetrad of haploid microspores which are held together by a callose wall, and ends with the degradation of the callose wall, and the release of individual microspores (Reynolds, 1997; Touraev *et al*., 1997). Microgametogenesis is the normal pathway of pollen development, and occurs with the continued growth of each microspore, which possesses a large central nucleus, ribosomes rich cytoplasm, and with various little vacuoles (Sangwan and Camefort, 1982) (Figure 1A-I). The single nucleus migrates to a peripheral position in the cell where undergoes an asymmetric division, the first haploid mitosis (Reynolds, 1997) (Figure 1A-II, III). This asymmetry of the mitotic fuse is due to the polarization of the nucleus, and consequently causes the unequal distribution of the cytoplasm in the daughter cells (La Cour, 1949).

The resulting bicellular structure, the pollen grain, consists of a small generative cell and a large vegetative cell (Figure 1A- IV). The first one is destined to divide once more in the second haploid mitosis, to form the two sperm cells involved in double fertilization. In most of the species, pollen grains are shed as two-celled entities whereas in families such as Asteraceae, Cruciferae and Gramineae the pollen contains three cells at the time of shedding (Reynolds, 1997). The final stage of microgametogenesis take place when the pollen grain germinates by extruding a pollen tube. This singular structure is capable of extended growth and serves to transport the sperm to the embryo sac.

1.3. An alternative route: androgenic pathways

Based on observations performed along the years on the abnormal development of pollen grains, the origin of pollen embryoids and calluses has been described for a wide diversity of plants. Four pathways are usually indicated through which microspores can embark into an embryogenic pathway instead of the normal pathway of gamete formation. The first one, known as A

pathway (Sunderland, 1973), starts with an asymmetric division of the microspore, giving origin, as in vivo, to a large vegetative cell and a the small generative cell. The first one continues to proliferate to give rise to an embryo or a callus (Sunderland, 1973; Dunweel and Sunderland, 1976a) (Figure 1B-V). This route is an odd kind of event, but it is one of the most common pathways of pollen embryogenesis and one that has been thoroughly investigated. As the initial divisions of the vegetative cell are under way, the generative cell either disintegrates or undergoes a few divisions (Raghavan, 1986). The A pathway has been described for a series of plants such as *Nicotiana tabacum* (Horner and Street, 1978), *Oryza sativa* (Chen, 1977), and many others.

In the second pathway, known as E pathway, the initial step is the same. However, it is the generative cell which proliferates and originates the multicellular pollen grains (Raghavan, 1986) (Figure 1B-IV). This route was first found by Raghavan (1978) in *Hyoscyamus niger*. The vegetative cell does not divide, or undergoes only a few divisions (Raghavan, 1986). This pathway is quite uncommon; however it has been observed in some species of cereals such as rice (Sun, 1978).

In *Datura innoxia*, which has been a favored material for the study of androgenesis, another situation has been described. After the asymmetric division, the vegetative cell and the generative cell, rather than embark on pathways of independent divisions, can both embark on the sporophytic pathway of development by starting to divide simultaneously and then fuse with each other after a few divisions (Figure 1B-VI). This pathway is known as C pathway and was initially described by Dunwell and Sunderland (1976b).

The last route, known as the B pathway, differs from all the above pathways. It starts with asymmetric division from which two morphologically identical cells arise. Further divisions of these cells produce a multicellular structure from which embryos or callus arise (Sunderland, 1973; Kaltchuk-Santos and Bodanese-Zanettini, 2002) (Figure 1B-VII). This pathway has been demonstrated in several species such as *Datura innoxia*, *Nicotiana tabacum*, and *Oryza sativa* (Raghavan, 1986).

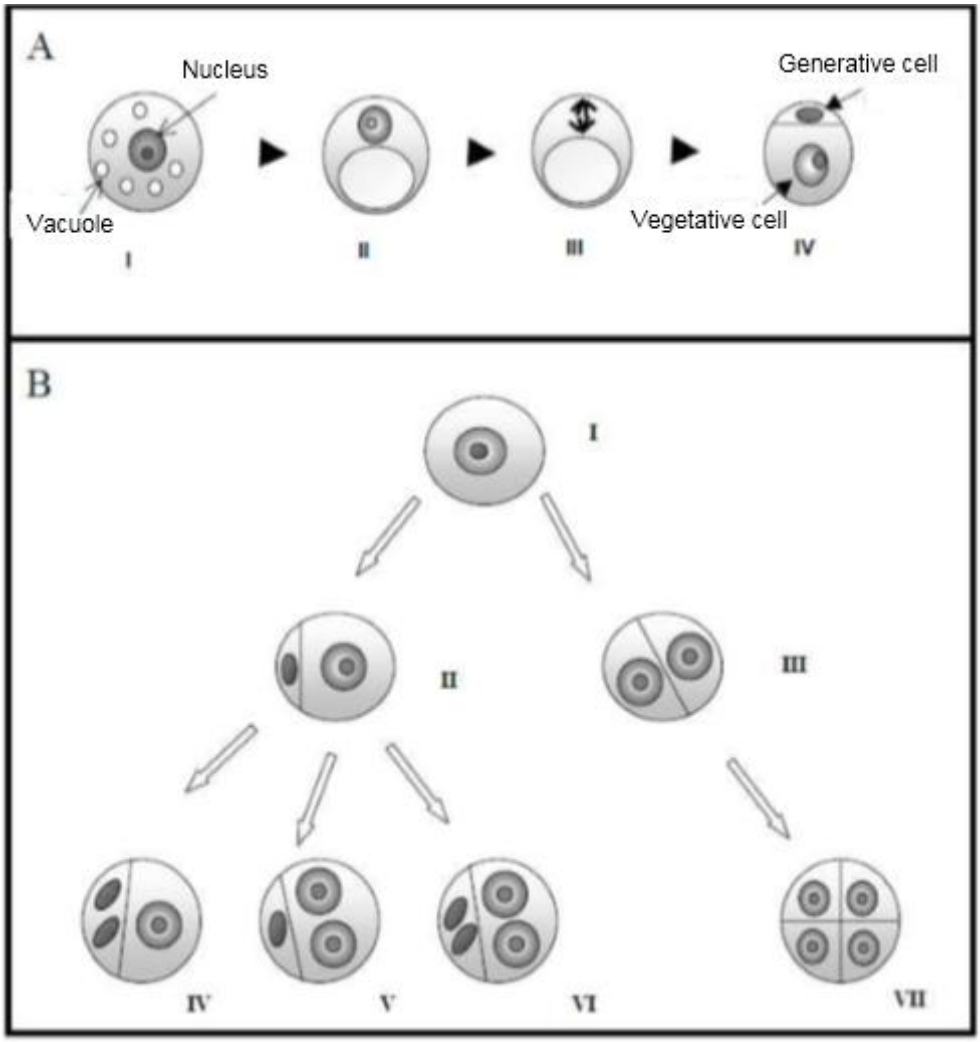

Figure 1 – **A. Microgametogenesis:** I. Young microspore with a central nucleus surrounded by small vacuoles; II. Vacuoles coalescence in a large one, the nucleus migrates to a peripheral position; III. Asymmetric mitosis; IV. Bicellular pollen grain with two structural and functional distinct cells, a small generative and a large vegetative cell. **B. Androgenic pathways *in vitro*:** I. Young microspore; II. Binucleate pollen; III. Binucleate pollen following a B mitosis; IV. E pathway – only the generative cell divides; V. A pathway – only the vegetative cell divides; VI. C pathway – both cells, vegetative and generative, divide; VII. B pathway – both cells continue to divide (adapted from Kaltchuk-Santos and Bodanese-Zanettini, 2002).

1.3.1. Androgenesis – The process

Regardless of the initial pattern, pollen grains suffer divisions until become multicellular, and in this stage there are two possible directions in the androgenic process to achieve the embryo stage: (1) the pollen acts like a zygote, undergoing various embryogenic stages resembling those of zygotic embryogenesis, the so-called **direct androgenesis**. This is observed in most of

the solanaceous and cruciferous species, e.g., *Datura, Nicotiana, Atropa, Brassica*; (2) multicellular pollen structures, instead of suffering normal embryogenesis, divide randomly to form a callus, which burst through the anther wall. This pathway is known as **indirect androgenesis**, and its common in species such as those of the genera *Asparagus* and *Vitis*, and among cereals (Sangwan and Sangwan-Norreel, 1987) (Figure 2). The callus either differentiates to form embryos or roots and shoots on the same medium, or it must be transferred to another medium to promote regeneration (Reinert and Bajaj, 1977).

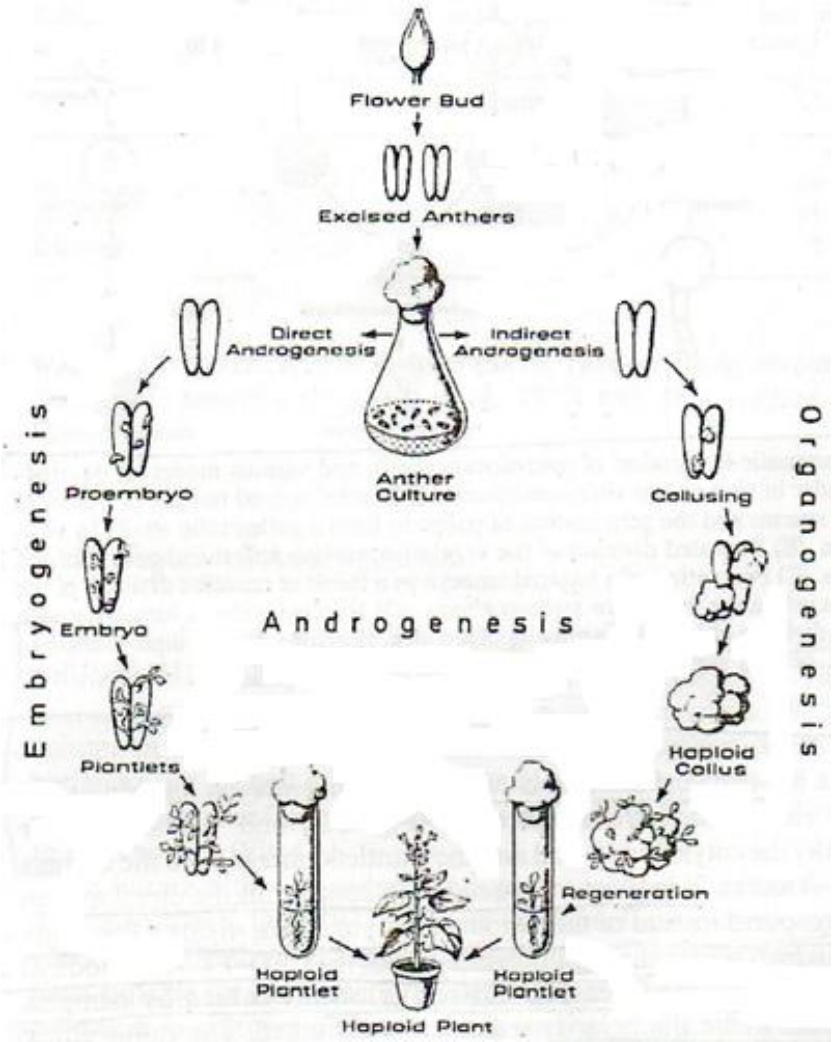

Figure 2 – Schematic representation of the culture of excised anthers and the two types of development of haploid plants by androgenesis, directly or through haploid callus (adapted from Reinert and Bajaj, 1977).

1.3.2. Androgenesis – Techniques

The techniques by which immature pollen is induced to divide and regenerate may be separated into two main types: **anther culture** and **microspore culture**. In the first one, the pollen is cultured still enclosed by the somatic anther tissues, and in the microspore culture technique these somatic tissues are removed and the pollen grains isolated and cultured (Dunwell, 1985).

a) Anther culture

This is the more commonly used technique to induced androgenesis in a number of species. It is a quite simple procedure in which anthers are removed from the plant and placed in a culture medium. Anthers are connected to the filament, and this one should be discarded from the culture to prevent somatic tissue callus, anthers have four pollen sacs where microspores are formed (Figure 3). A number of the microspores will survive and follow the androgenic pathway of development (Bajaj, 1983; Dunwell, 1985; Wedzony *et al*., 2009).

Young flower buds at the appropriate stage of pollen development are surface-sterilized and the intact, uninjured dissected anthers inoculated on nutrient media (Narayanaswamy and George, 1982). During dissection, care should be taken to avoid injury to anthers as they often tend to produce callus from the injured surface, from the anther wall, so this anthers should be discarded (Maheshwari *et al*., 1980; Bajaj, 1983). Excised anthers must be placed in culture in direct contact with the nutrient medium as described by Sopory and Maheshwari (1976a), they found that placing the anthers horizontally on the surface of the medium resulted in the formation of the largest number of embryoids and that immersion of even a portion of the anther in the agar medium reduces anther response.

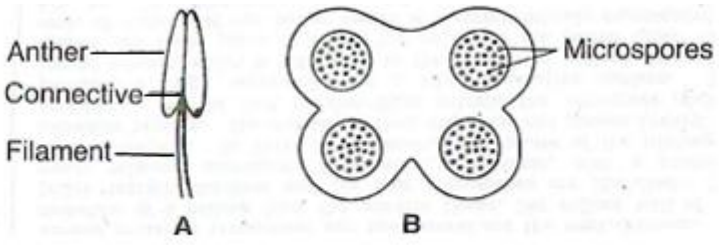

Figure 3 – A. A stamen comprising filament, connective tissue and anther. B. An outline of a cross section of an anther showing four pollen sacs containing uninucleate microspores (adapted from Bajaj, 1983).

Anther culture is often the method of choice for haploid production in many crops because the simplicity of the approach allows large-scale anther culture establishment and application to a wide range of genotypes, and it was been widely used along the last years (Germanà, 2011). This technique is most used is cereals being a fast method for production of haploid or dihaploid plants that are used in breeding purposes, as an alternative to the numerous cycles of inbreeding or backcrossing usually needed to obtain pure lines in conventional plant breeding, becoming a well-established routine method in commercial plant breeding for some cereals (Jähne and Lörz, 1995).

b) Microspore culture

Anther culture technique suffers from one main disadvantage: plants may originate not only from the pollen grains, but also from various parts of the anther. The result can be a mixed population of plants with various levels of ploidy and different origins difficult to distinguish (Maheshwari *et al*., 1980). Furthermore its application to various crop plants is limited and, considering the large number of pollen grains in the anther, yields are still very low (Heberle-Bors, 1989). To overcome these drawbacks a new method has emerged: the culture of isolated microspores.

Microspore culture offers the advantage that the sporophytic anther wall tissues do not interfere in the process, and the development of the embryo can thus be followed directly (Touraev *et al*., 1997; Davies, 2003). The first success in culturing microspores free of the surrounding anther tissue was reported by Nitsch and Norreel (1973) in *Datura innoxia*. The technique of regenerating fertile plants from isolated microspores represents a potential tool for different

biotechnological applications. Microspores are very promising targets for genetic manipulation, because they are unicellular and can be regenerated to homozygous plants (Jähne and Lörz, 1995). The technique consists in the isolation of the microspores by gently squashing the anthers, in a liquid medium. Then the suspension has to be washed to eliminate the somatic tissues of the anther and then finally inoculate Petri dishes with the final suspension. Several methods for separating pollen from somatic tissues have been developed. (Dunwell, 1985; Sangwan and Sangwan-Norreel, 1987).

This technique offers the unique advantage of allow the sequence of androgenic events to be observed starting from a single cell, and thus more suitable for understanding the physiology and biochemistry of androgenesis, and the *in vitro* factors, that can directly affect the pollen behavior and haploid plant formation (Reinert *et al*., 1975; Bajaj, 1983; Heberle-Bors, 1989). Although microspores culture is an improvement over the anther culture method, only limited success has so far been achieved using this approach, due to the difficulty of the technique that requires higher skills and better equipment than anther cultures (Heberle-Bors, 1989). The need of an intermediate step of anther culture before microspore isolation to increase the efficiency of the process indicates that the anther wall may play a critical role in the early stages of development (Bajaj, 1983).

1.4. Factors affecting androgenesis

Androgenesis can be induced under adequate conditions, however there are numerous factors that influence the process and its efficiency, such as the physiological status of the anther donor plants, the genotype, the developmental stage of the microspores at the time of culture, the *in vitro* culture conditions, and the application of stress pretreatments.

Also, the information about the mechanisms underlying induction is scarce, nonetheless studies have been shown that this redirection requires a stimulus-signal (Garrido *et al*., 1993). In anther culture systems stress is of critical relevance for blocking gametophytic development and for triggering androgenesis in competent microspores (Immonen and Robinson, 2000).

a) Growth of donor plants

The quality of donor plants is a key factor for the success of androgenesis. The sample of microspores, their release from the anther, and their subsequent divisions leading to plant regeneration often depend on the conditions under which the donor plants grow in a particular environment (Datta, 2005). Experiments have shown that the number of microspores capable of division and regeneration can vary widely within a variety due to the environmental conditions in which the donor plants are grown (Vasil, 1980; Jähne and Lörz, 1995).

The main environmental factors affecting the vigor of donor plants include light intensity and quality, nutrition, photoperiod and temperature (Zheng, 2003). In order to establish a reproducible culture system the donor plants should be grown under controlled conditions either in a greenhouse or in a phytotron (Jähne and Lörz, 1995). Although well-ordered conditions seem to be essential for a controlled androgenesis, in general plants grown in the field have shown to be more effective as donors of anther or pollen (Pandey, 1973; Vasil, 1980).

Seasonal variation in anther response is common in a number of species, therefore the age of the donor plants is an important aspect in androgenesis. In rice, plants grown during the dry season have provided the best microspore response (Datta, 2005).

b) Genotype

One of the most significant factors controlling the success of *in vitro* induction of haploids is plant genotype. It has been repeatedly observed that various species and cultivars exhibit different growth responses in culture (Bajaj, 1983, Datta, 2005).

One of these reports is the one made by Guha-Mukherjee (1973) that observed a strong effect of the genotype in the rice androgenesis. The occurrence of a strong genotype specificity is a general feature for all cereals. Obviously, genetic factors are important in determining the level of anther and microspore culture response (Jähne and Lörz, 1995).

c) Microspore Stage

In general, there are two distinct phases of pollen development more adequate for switching pollen towards a sporophytic pathway: just before and immediately after the first haploid mitosis. The first haploid mitosis marks the end of the “juvenile” or “uncommitted” period of the pollen. Before this division the microspore has a large central vacuole, a thin-layer cytoplasm with few undifferentiated organelles. After this unequal asymmetric division, pollen enters into the maturation or gametophytic phase. Thus, the first haploid mitosis can serve as a marker, indicating the end of a dedifferentiated or a reversible state, and the beginning of a differentiated or an irreversible state (Sangwan and Sangwan-Norreel, 1987).

The formation of atypical pollen grains lacking the usual generative-within-vegetative cell arrangement has been reported in anther cultures of several species. These structures, designated P-grains by several authors, provide recognized pathways of androgenesis and are thought by some researchers to be the principal route in the formation of haploid embryos (Nitsch and Norreel, 1973; Dunwell and Sunderland, 1976c). These P-grains are usually smaller, uni or bi-nucleated with a thin exine (Kaltchuk-Santos and Bodanese-Zanettini, 2002). Horner and Street (1978) reported that all grains in *Nicotiana tabacum* that underwent androgenesis were of this type.

The production of embryos from the haploid microspore represents a very dramatic switch in development that can only be achieved in the cells during a brief period immediately prior to, and following, the first pollen mitosis (Zaki and Dickinson, 1990). The significance of the age of the pollen in anther culture has been widely discussed. The critical stage varies from species to species. Generally, pollen cultured just prior to or after the first mitosis gave a positive response by producing typical embryos or callus masses, opposed to those showing meiotic stages in pollen mother cells and older ones with starch-filled grains that failed to produce embryos (Narayanaswamy and George, 1982). Observations on induced growth of anthers in several species seem to suggest that the most responsive phase for an anther to produce embryoids or callus is when microspores are at the uninucleate stage (Pandey, 1973).

In *N. tabacum* the number of anthers producing plantlets increased as the pollen at the time of anther excision approached the first mitosis, and a maximum response was shown by anthers containing early bicellular grains (Horner and Street, 1978, Garrido *et al*., 1993). On the other hand, in cereals and *Brassica* species the optimum stage is the early uninucleate period (Dunwell, 1985). It seems then that the stages most responsive to androgenic induction are either mid- to late-uninucleate or early-binucleate (Raghavan, 1986; Reynolds, 1997) (Figure 4).

Although the growth conditions influence the morphology of the donor plants it is possible to correlate the developmental phase of a tiller to the developmental stage of the microspores. Usually spikes can be preselected on the basis of the inteligule length between the flag leaf and the second leaf, and on the thickness of the tiller (Jähne and Lörz, 1995).

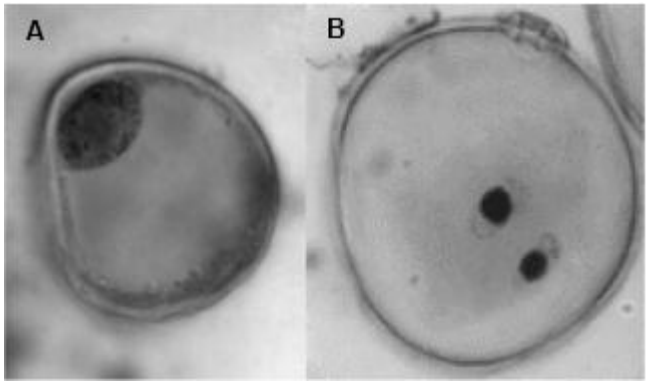

Figure 4. A. Late-uninucleate microspore. B. Early-binucleate microspore.

d) Culture conditions

Many efforts have been made to optimize culture media for androgenesis induction, especially in cereals. The induction medium has as a role not only microspore nourishment, but also to direct the pathway of embryo development (Jähne and Lörz, 1995; Datta, 2005). Thus, the composition of the medium is one of the most important factors determining not only the success of anther culture but also the mode of development (Bajaj, 1983). Generally, an induction medium contains macro and microelements, vitamins, sugars, and growth regulators. Sucrose (or other carbon source) is indispensable in the medium, because it acts both as a carbon source and as an osmoregulator compound

decreasing the water potential of the environment where the anthers/polle are present (Vasil, 1980; Bajaj, 1983; Hu and Zheng, 1984). For most species, 2 to 3% sucrose in the media is adequate, although in some cases a higher concentration has been used. In cereals, the induction of haploid embryoids requires a high sucrose content and a corresponding high osmotic pressure in the culture medium (Heszky and Mesch, 1976). The nutritional requirements vary greatly among species (Narayanaswamy and George, 1982).

Chu *et al*. (1975) found that high concentrations of ammonium ions inhibit pollen callus formation in rice showing the role of this nutrient on pollen embryogenesis (Datta, 2005). Based on this premise, the N6 medium with reduced concentration of ammonium ions and with a modified ammonium to nitrate ratio was developed and proved to be more efficient than other synthetic media for anther culture of rice and as well as in other cereals (Hu and Zheng, 1984). Since then on, two basal media, the chemical defined N6 medium and the Murashige and Skoog (1962) medium, have been generally used with modifications (Datta, 2005).

Over the years, some studies reported the apparent toxic effects of sucrose on cultured microspores (Scoot and Lyne, 1994). In some studies maltose has been used as an alternative carbon source with promising results in species such as wheat (Last and Brettell, 1990; Scott and Lyne, 1994).

PGRs, particularly auxins and cytokinins, have been successfully used for the induction of androgenic development. Anthers of many cereals, such as barley and rice, require auxins. The presence of auxins in the medium promotes rapid cell proliferation and subsequent callus formation (Vasil, 1980, Sangwan and Sangwan-Norreel, 1987). However, media rich in growth regulators encourage the proliferation of tissues other than microspores, like anther wall or filament, and should be avoided, because in such cases mixed calli with cells of different ploidy levels may be obtained (Reinert and Bajaj, 1977). Indole-3-acetic acid may induce direct embryogenesis, whereas 2,4-Dichlorophenoxyacetic acid (2,4-D) favors rapid cell proliferation and callus formation. When 2,4-D is replaced by α-naphtaleneacetic acid (NAA), a weaker auxin, haploid plants are formed in the induction medium without the need of callus transfer to a regeneration medium (Ball *et al*., 1993).

An adequate balance of exogenous hormones in the culture medium is crucial for both the yield and quality of embryos. Although there is no general agreement on the optimal PGRs concentrations and combinations, the consensus is to use the lowest possible concentrations, since higher PGRs levels in induction culture are generally detrimental to plant production as cell division and differentiation shift towards calli rather than embryos (Zheng, 2003). It seems that the presence of growth hormones always increased the percentage of responding anthers unless they were added in very low or very high concentrations (Sopory and Maheshwari, 1976b). Even though auxins and cytokinins are the two types of PGRs most widely used in media for anther/microspore cultures, cytokinins were found to be the best in favoring the production of embryoids (Sopory and Maheshwari, 1976b; Zheng, 2003).

Other important conditions affecting androgenesis arelight and temperature. Anther and microspore cultures are usually incubated at 25ºC, but in many species an improved response is obtained by increasing the temperature to 30ºC (Vasil, 1980). Light is known to be required for the induction of androgenic development. However the effect or even the requirement of light for haploid production is not clearly understood, varying from species to species (Vasil, 1980). In most cereals, incubation of cultures in darkness promoted callus growth from microspores, but regeneration of plantlets occurred only when cultures were transferred to light (Narayanaswamy and George, 1982).

e) Stress pretreatments

Several lines of research have shown to be important to directing the microspores towards embryogenesis. The deviation from a gametophytic to a sporophytic developmental pathway has been induced in microspores by applying various pretreatments either *in vivo* or *in vitro* (Touraev *et al*., 1997; Immonen and Robinson, 2000). Stress can be the signal to initiate the androgenic process, since stress proteins have been detected during the induction process, indicating that these proteins may be involved in cell reprogramming (Kiviharju and Pehu, 1998).

There are a number of pretreatments that are known to trigger androgenesis, such as high or low temperature. Starvation and colchicine

treatment are two of the most common stresses applied at the time of culture and have shown to play a vital role in the reprogramming of microspores, repressing the gametophytic pathway of microspores to fertile pollen, which leads to an intermediate stage of dedifferentiation and cell totipotency (Islam and Tuteja, 2012). Although these pretreatments can induce androgenesis, the type and duration of pretreatment varies according to species and type of explant (Roberts-Oehlschlager and Dunwell, 1990).

Anther wall seems to act as some kind of barrier to the flow of nutrients from the culture medium to the microspores, and this starvation apparently stimulates the abnormal divisions of microspores triggering androgenesis (Heberle-Bros, 1989). Another treatment that has been shown to be a powerful factor to promote androgenesis is colchicine. This c-mitotic agent is the most frequently drug used for chromosome doubling in plants. It acts by blocking tubulin polymerization and spindle function during mitosis disturbing normal polar segregation of sister chromatids. The result is the formation of a restitution nucleus containing twice the number of chromosomes (Levan, 1938; Zhao *et al*., 1996; Hansen and Andersen, 1998). According to Zaki and Dickinson (1990), application of colchicine before the first mitosis resulted in a significant increase in the number of cells undergoing B-mitosis and androgenesis.

When this drug is used in low concentrations it only inhibits cell division for a short time, therefore stimulates a symmetric division which consequently increase the frequency of embryos of microspore origin. However in high concentrations the drug has a toxic effect that reduces the frequency of androgenesis (Hansen and Andersen, 1998; Barnabás *et al*., 2001). Colchicine application and the comprehension of its beneficial effects on androgenesis needs to be clarified by further studies.

Temperature-stress pretreatment is a means of enhancing the production of pollen plants from cultured anthers. Several studies have shown that the efficacy of the pretreatment, when applied to excised flower buds prior to culture of the anthers, depends upon various factors, such as the temperature and the duration of the treatment, plant age, the developmental stage of the anthers and genotype. There was significant genotypic variation in the response of anther culture to temperature, and growth conditions of the plants, given that, even anthers from the same genotype may give different culture responses if the

donor plants are grown under different conditions (Huang and Sunderland, 1982; Ouyang *et al*., 1987). Temperature treatments, hot or cold, or a combination of both, may cause different responses in different species (Pandey, 1973).

High temperature seemed to trigger or at least enhance androgenesis (Baillie *et al*., 1992; Custers *et al*., 1994). For example, in wheat and tocacco high temperatures (32-34ºC) pretreatment applied to anthers or isolated microspores increases callus and embryoid yields (Ouyang *et al*., 1987; Zheng, 2003). The effects of heat treatments in androgenesis were investigated over the years, and Binarova *et al*. (1997) revealed the presence of heat-shock proteins (HSPs), and a high correlation between the androgenesis and HSPs synthesis was also found (Cordewener *et al*., 1995).

One of the most applied and successful pretreatments used is cold pretreatment. This approach has been used in a variety of plant species for induction of androgenesis (Jähne and Lörz, 1995). Cold incubation of excised flower buds before culture has been used to increase the frequency of androgenesis. In wheat the response increased two times when anthers were pretreated at 1-4ºC for 48 hours; in rice and other cereals cold pretreatment proved also to be favorable (Hu and Zheng, 1984). Thermal shocks (3-4ºC for 2 days) given to anthers before culturing encouraged equal division of the microspore nucleus, which eventually resulted in about 10 % increase in androgenesis (Bajaj et al, 1977). Nitsch and Norreel (1973) found an increased response of *D. innoxia* anthers that were taken from excised flower buds stored for 48 hours in a refrigerator. These initial observations have been confirmed by several other studies, and the cold preatreatment is now used extensively (Sangwan-Norreel, 1977; Camefort and Sangwan, 1979; Sunderland and Roberts, 1979).

Chilling of anthers prior to culture increases the number of microspores that undergo an atypical mitosis, resulting in the formation of two equal and/or identical cells instead of the distinct and characteristic generative and vegetative cells (Nitsch and Norreel, 1973; Maheshwari *et al*., 1980). The exposure of anthers to low temperatures for 2-3 days drastically reduces their metabolic activity, which possibly results in the accumulation of a larger percentage of microspores at the most suitable stage for effecting the

developmental shift required for androgenic development (Vasil, 1980). Cold pretreatment may act in different ways: delaying the first haploid mitosis, inducing the formation of two equal nuclei, increasing the viability of competent microspores, increasing the permeability of the pollen wall, or modifying the microspore wall through the disorganization of the tapetum (Sangwan and Sangwan-Norreel, 1987). It seems that the cold pretreatment does not induce androgenesis, but it enhances the viability of cultured pollen, and cause repression of the gametophytic differentiation which results in higher frequency of androgenesis. Hence the effect of cold pretreatment is indirect (Bajaj, 1983). Therefore cold incubation of donor spikes prior to culture may have a dual effect by interrupting normal gametophytic development and allowing sufficient time for the embryo to be nursed by the anther (Mejza *et al*., 1993; Zheng, 2003).

As referred in the previous sections, rice is one of the most used plants for haploids formation through anther or pollen culture, and with some considerable success over the years. This is particularly important since rice is one of the most cultured cereal crops in the world which means that all the scientific improvements of this species may have a positive impact on food production.

1.5. Rice (*Oryza sativa* L.)

1.5.1. Characterization of the world's most important crop plant

Rice (*Oryza sativa* L.; 2n = 2x = 24) belongs to family Poaceae and to the genus *Oryza* which includes 24 recognized species. Two of them, *O. sativa* and *O. glaberrima* Steud., are species used in agriculture, the first one in many countries but particularly in Asia, and the second one mostly in Africa (Gosal *et al*., 1997). The genus *Oryza* seems to have originated about 130 million years ago in the super continent Gondwanaland that eventually broke up and drifted apart become Asia, Africa, The Americas, Australia and Antarctica (Khush, 1997).

Rice is an annual grass, its culm is erect, cylindrical and smooth slightly swollen at the node which is solid but hollow in the internode. The leaves are distichous and the base of each ligulate leaf forms a tube (sheath), completely enclosing the whole of the upper internode but split down the side opposite the

blade except at the node of its insertion (Majumdar and Saha, 1956) (Figure 5). The rice grain, a caryopsis, is a dry one-seeded fruit in which the seed occupies all the fruit and the pericarp is fused to seed coat. (Skider *et al*., 2006). Rice is the world's single most important food crop and a primary food source for more than a third of the world's population. More than 90% of the world's rice is grown and consumed in Asia where 60% of the earth's people live. Rice accounts for 35 to 65% of the calories consumed by 3 billion Asians (Figure 6) (FAO). Rice is the only major cereal crop that is consumed almost exclusively by humans and it's planted on about 148 million hectares annually, or on 11% of the world´s cultivated land (Khush, 1997). Keeping in view the annual average population growth rate of approximately 1,5% and estimated per capita consumption of about 250 g of rice per day, the demand for rice is expected to increase 40% by 2025 (Khush, 2004).

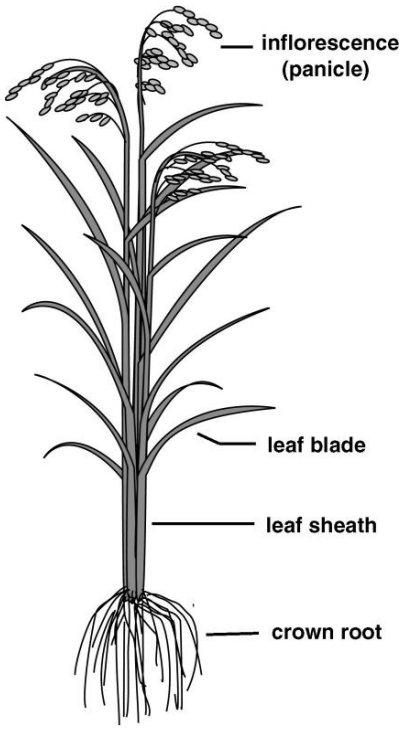

Figure 5 – Schematic representation of a rice plant (adapted from Itoh *et al*., 2005).

All the rice cultivars grown in Asia, Europe and America are *O. sativa*, whereas many of those cultivated in West Africa are *O. glaberrima*. It is probable that domestication of wild rice started about 9000 years ago in Asia. *O. sativa* consists of two distinct subspecies, recognized first by the Chinese since the Han dynasty, the *indica*, or Hsien, and *japonica*, or Keng. The

supspecies *indica* is grown in the humid tropics whereas *japonica* cultivars are cultivated in Japan and Northern China under cooler climate. The Portuguese introduced tropical *japonica* cultivars and lowland *indica* cultivars in Brazil and Spanish people brought them to other Latin American countries. The distinction between *indica* and *japonica* is partly based on the existence of reproductive barriers between them (Gosal *et al*., 1997; Khush, 1997). Rice is grown under diverse growing conditions. Four major ecosystems are generally recognized as follows: irrigated, rainfed lowland, upland and floodprone. Approximately 55% of the world rice area is irrigated (Khush, 1997).

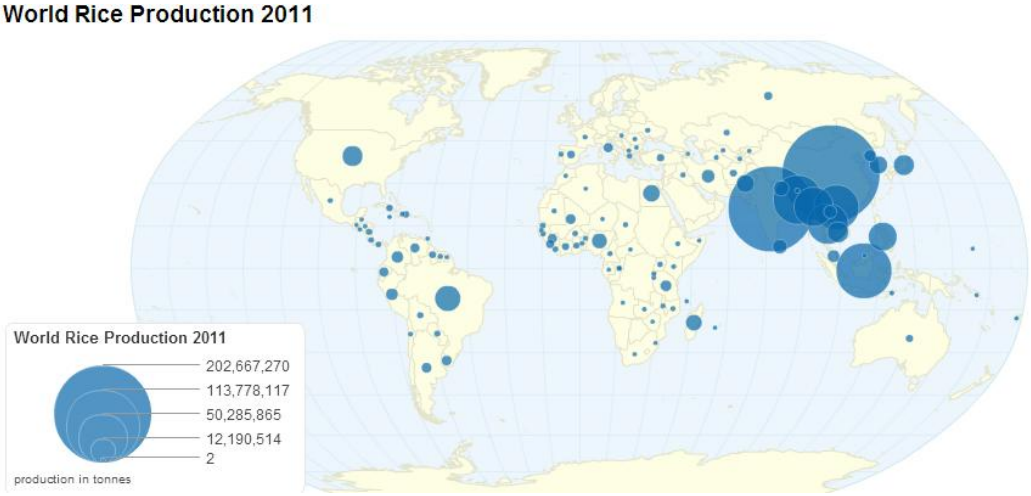

Figure 6 – World rice production in 2011, where it's obvious the Asia supremacy, representing the major areas of production, with more than 200 million tons of rice produced in only a year (adapted from FAO, http://faostat.fao.org).

During the 1950 and 1960, the rapidly increasing world population caused great concern about the availability of sufficient food to forestall massive starvation, so in 1960, a group of forward-looking leaders of the Rockfeller Foundation and the Ford Foundation decided to establish an institution which the main objective would be to improve the production efficiency of rice and to do this for the benefit or rice farmers and consumers. In this context IRRI (International Rice Research Institute) was born with the support of the Philippines government (Khush, 1997). A considerable improvement of rice has been done through traditional rice breeding, and higher yielding, semi-dwarf, photoperiod-insensitive and pest resistant cultivars emerged (Gosal *et al*., 1997; Skider *et al*., 2006). To meet the global demand for rice consumption, there is an urgent need for rapid development of even higher yielding rice.

Biotechnology offers great potential for production, conservation, characterization and utilization of germplasm for rice breeding programs. By using biotechnological techniques, such as anther culture, more than 100 new rice cultivars were already developed (Gosal *et al*., 1997).

Rice has become, over the years, a model plant in anther culture improvement, not only by its immense economic and nutritional importance, but also because it has a relatively small genome (~430Mb), the smallest between the major crop plants, which has been completely sequenced and it can be genetically modified by various transformation methods (International Rice Genome Sequencing Project, 2005; Summart *et al*., 2008).

1.5.2. Anther culture in rice

Anther culture, is an important technique for immediate fixation of homozygosity thereby reducing the breeding cycles and providing opportunity to recessive genes to be full expressed (Sah and Niroula, 2007). After the discover by Guha and Maheshwari (1964) in *Datura*, several other researchers attempt to reach the same success in other species, and four years later Japanese researchers Niizeki and Oono (1968), first demonstrated that haploid plants of rice could be obtained from *in vitro* culture of immature anthers. They showed that when anthers of rice were cultured on a medium supplemented with IAA, 2,4-D and kinetin, multicellular bodies appear from within the anther in about 4 to 8 weeks. These later yield a dense callus. Cytological examination of the callus confirmed its haploid nature and its origin from pollen grains. These results were further confirmed by Iyer and Raina (1972).

After this discovery several studies succeeded to achieve haploid plants from anther culture in both *indica* and *japonica* cultivars. However the *indica* has shown to be more difficult to regenerate via callus culture (Lee *et al*., 2000).

In rice anther culture, a microspore can be induced to form a plantlet by callus or embryo pathway which is determined mainly by the concentration and combination of hormones in the induction medium (Ying, 1986). In hormone-free media microspores give rise to plantlets directly, but the induction frequency is rather low (Ying, 1986). When a higher concentration of 2,4-D is used as exogenous hormone, a microspore is usually induced to form callus

which subsequently differentiates into a plantlet after being transferred to a medium without 2,4-D (Ying, 1986). There are two pathways in rice androgenesis. In the first pollen division is unequal and forms two cells: vegetative and generative. The vegetative or the generative cell kept on dividing, and forms callus/embryos. In the second route the first pollen division is equal leading to two identical cells (Gosal *et al*., 1997).

In rice, factors affecting androgenesis are various, such as pollen stage, medium, incubation condition, physiological sate of donor plant, and genotype. The developmental stage of pollen at the time of anther inoculation is a key factor (Cornejo-Martin and Primo-Millo, 1981). Comparative studies have indicated that callus induction was highest when anthers containing microspores in mid-uninucleate stage were used. However, early uninucleate stage, late unicleate stage and bi-nucleate stage have been also reported to be involved in the androgenic pathway (Chen, 1977; Cornejo-Martin and Primo-Millo, 1981; Ying, 1986). Usually boots are collected from the field when the distance between the base of the flag leaf and the auricle of the last leaf is 3-8 cm, depending upon the cultivar. The middle part of the collected panicle contains microspores at the uninucleate stage, therefore those anthers should be used (Gosal *et al*., 1997). Other criteria can be used, such as the color and size of spikelets and anthers (Chen and Chen, 1979).

Culture medium is another important factor controlling rice androgenesis. Since Chu *et al*. (1975) developed the N6 medium, with low levels of ammonium, specifically for rice anther culture that this medium has been demonstrated to be the most suitable as induction medium (Chen *et al*., 1982a; Tsay *et al*., 1982). Chen (1978) reported that the rate and subsequent organogenesis increased as the sucrose concentration increased from 3 to 9%; however, calli initiated on a medium with 9% sucrose regenerated more albino plantlets. A combination of 6% sucrose in the callus induction medium and 3% in the plant regeneration medium gave the highest frequency of regenerated green plants, and it has also been suggested that optimal sucrose concentration for callus induction is 3% (Kavi Kishor *et al*., 1989). Chaleff and Stolarz (1981) suggested that the influence of sugar concentration on callus formation may be its effect on the water potential of the medium rather than its utilization as a carbon source.

The formation of callus is regulated by the type and level of the auxin in the culture medium (Trejo-Tapia *et al*., 2002). A high auxin concentration causes dedifferentiation in rice tissues, while the removal of an auxin causes the differentiation or redifferentiation (Nishi *et al*., 1973). A combination of auxins, such 2,4-D, NAA, IAA and cytokinins, such as kinetin has been widely used in rice anther culture (Gosal *et al*., 1997). Reddy *et al*. (1985) reported that NAA was better to induce callus, but 2,4-D was better for plantlet regeneration of *indica* cultivars. However, high amounts of either NAA or 2,4-D enhanced callus formation and simultaneously reduced plant regeneration. The addition of NAA alone to the regeneration medium resulted in regeneration inhibition, but when NAA is added together with kinetin, a substantial degree of regeneration was observed (Tsukahara and Hirosawa, 1992). Nevertheless, the addition of 2,4-D completely inhibits the organogenesis of the calli (Cornejo-Martin and Primo-Millo, 1981). Reddy *et al*. (1985) have shown that both 2,4-D and NAA at high concentrations increased callus induction but plantlet yield was poor. Chen *et al*. (2001) showed that 2,4-D promoted the formation of callus in anthers of rice, but in concentrations higher than 10 µM the induction started to decline (Figure 7). Results shared by Gupta *et al*. (1989) in 2,4-D and kinetin, indicating a possible synergism between auxins and cytokinins in embryogenic callus induction.

As Maheshwari *et al*. (1980) stated, certain physical and chemical treatments given to flower buds or anthers prior to culture, can be highly inductive. The most significant is cold treatment. According to Genovesi and Magill (1982), rice cold treated anthers containing uninucleate microspores form callus at a much higher rate. The beneficial effects of cold treatment in rice have been reported by several studies (Ying *et al*., 1982; Zapata *et al*., 1982; Sathish *et al*., 1995, Xie *et al*., 1997). The most common treatment is to submit boots at 4ºC for 7-10 days (Gosal *et al*., 1997). For optimal anther culture response, the cold pretreatment requirement depends on the cultivar (Cho and Zapata, 1980).

Thanks to anther culture, new rice varieties, including “Huayu Nº1” a more yielding and resistant variety, were developed in China, opening the doors for new cereal varieties that can now be developed in four or five years, and their breeding cycle is much shorter than that in the past. Hence, haploid breeding

proves in practice to be a new and effective technique for improvement crops (Han *et al*., 1978).

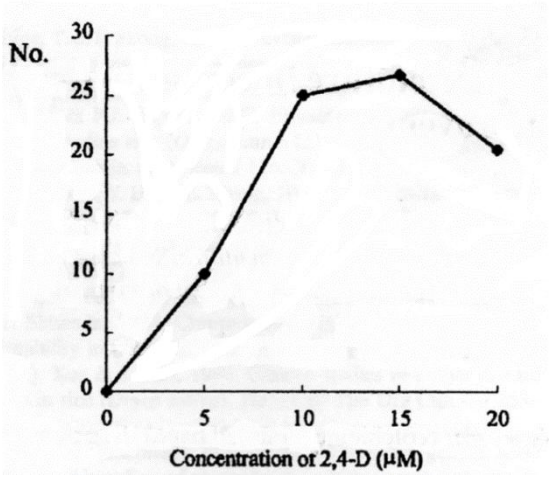

Figure 7 – Relationship between the number of calli formed (No./100 anthers) and concentration of 2,4-D supplemented in the induction medium (adapted from Chen *et al*. 2001).

1.5.3. Albinism

The greatest problem in androgenesis of cereals, is the formation chlorophyll-deficient plantlets usually known as albinos (Brettel *et al*., 1981). It is not uncommon that more than 90% of the regenerated plantlets could be albinos, with strong variations among genotypes (Ying, 1986). It has been found that the albinos, at least in barley and rice, do contain plastids or protoplastids indicating that they are not likely to have developed from the generative cell. It has also been reported that in rice albinos the protoplastids are devoid of rRNA and the large subunit of the fraction-1 protein, which is coded by chloroplastidial DNA (Vasil, 1980). Four main possibilities have been proposed to explain the high rates of albinos formation: (1) the albino plants are derived either from genetically deficient or from dimorphic pollen; (2) the culture procedure induces a genetic change in embryogenic pollen, leading to permanent destruction of chloroplastidial information; (3) a purely physiological basis due to unsuitable culture conditions, such as temperature; (4) abnormalities during cytokinesis lead to production of micronuclei which are somehow linked to inhibition of chlorophyll synthesis. This means that the cause of the albinism can be genetic physiologic or both (Dunwell, 1978).

1.5.4. Ploidy

It must not be assumed that the products of anther or microspore culture, are haploid plants. In many species haploids are even the exception and ploidies up to hexaploidy have been found (Dunwell, 1985). The wide range of ploidy levels seen in androgenic plants has been attributed to endomitosis and/or the fusion of various nuclei, with the first one causing diploidy, whereas the second would give rise to triploids and pentaploids (Vasil, 1980). In rice, plants ranging from haploid to pentaploid have been found; however, haploids and diploids are generally the most common (Chen *et al*., 1982b). It has been observed that cells in culture exhibit cytological instability. Moreover, it has been established that haploid cell lines have a greater tendency to increase in ploidy level to diploid, this tendency is a major obstacle to the maintenance of haploid cells in culture (Vasil, 1980; Bajaj, 1983).

One of the most common techniques of doubling of the chromosome set in plants is by using colchicine. This technique has been employed for obtaining homozygous diploid plants from haploid cultures (Bajaj, 1983). However, the toxic effects of the drug and the need to control the concentrations and time of application make necessary the search for an alternative technique.

The diploid plants in general appear to arise from the somatic anther tissue, although some could be also originated from the fusion of pollen nuclei. The embryos and plants obtained from such pollen would be completely homozygous, this spontaneous chromosome doubling reduces the chances of nuclear aberrations in diploid cells, making this feature one of the most important attributes of anther culture (Vasil, 1980; Bajaj, 1983). Spontaneous chromosome doubling is frequently observed in rice haploids, being assessed by the formation of fertile panicles (Reiffers and Freire, 1990).

It is well-known that chromosome numbers and ploidy level are highly variable in regenerated plants derived from callus. The resulting plants by anther culture are not only haploid but also diploid, and some plants become polyploids or aneuploids. Generally, the diploid plants are named doubled-haploid and, in rice, were first obtained by Niizeki and Oono (1968). Although the ploidy levels can be identified by analyses of morphological features such as fertility, culm length, glume length, awns and other phenotypic characters, a

more reliable method, allowing for a better evaluation of the DNA content of the regenerated plant is required. Flow cytometry is this method and during the last years it has become a very important tool for ploidy analysis.

1.6. Main goals

In Portugal, rice culture dates back to the VIII century (Bajaj, 1991). And, nowadays the country leads rice consumption in Europe with values greater than 17kg/person/year (EUROSTAT) The Mondego valley is one of the most important regions for rice production together with the valleys of the rivers Sado and Tejo, in the South. However, Portugal rice improvement has been made mostly by conventional breeding methods, opposed to the international scenario, where great advances have been obtained by using biotechnological approaches (Uchimiya and Toriyama, 1991). These advances will make substantial contributions to crop improvement and human nutrition (Bhullan and Gruissem, 2013).

The main objective of this work was to test the androgenic potential of several cultivars usually grown in the Mondego valley and also of genotypes of a breeding program which is being developed by the INIAV and ITQB. All the genotypes used were growing at the experimental field of Bico da Barca, near Montemor-o-Velho (40º 10’ 37.39’’ N, 8º 39’ 44.36’’ W). In this experimental field the cultivars are selected through pedigree method, which consists in the selection of plants from F2 generation and the subsequent generation and testing their progenies.

The effect of culture media (induction and regeneration) and the levels of ploidy of the regenerated plantlets were analyzed. Other of the objectives was to obtain seeds from the pollen derived plants for further studies in the scope of the breeding program above mentioned.

2. Materials and Methods

Materials and methods

2.1. Plant material

The anthers of rice used in this work were gathered from plants growing in the experimental field of Bico-da-Barca, Montemor-o-Velho (Regional Direction of Agriculture and Fisheries of the Center), in the Mondego valley (Figure 8 and 9). All rice varieties and breeding lines used in this work belong to the sub-species *japonica*. The experimental lines cultured in this field are selected through the pedigree breeding method.

The first assays were carried out in 2010, in which four rice varieties, Ariete, Dardo, Eurosis and Sirio, and six experimental F3 breeding lines, 2501, 2503, 2506, 2509, 2531 and 2536 were tested. In 2011, another four varieties, Ariete, Eurosis, Opale and Ronaldo; three F2 lines, 1131, 1133 and 1134; and three F3 lines, 2520, 2524 and 2527 were used. Finally, in 2012, the same four varieties of 2012 were used, and four F3 lines, 2503, 2510, 2515 and 2523 (former F2 line 1134), and four F4 lines, 3514 and 3515 (formers F3 line 2520), and 3521 and 3523 (formers F3 line 2527), were assayed, according the scheme of figure 10.

Figure 8 – Google earth image from the rice culture fields in Mondego valley with Bico da Barca experimental field indicated by the red limits.

Figure 9 – Rice fields from Bico da Barca experimental fields.

The flowering tillers were all collected in the field in the last days of July, when the base of the flag leaf and the auricle of the last leaf was 3-8 cm length depending upon the cultivar, prior to the emergence of the panicles from the flag leaf sheath, as described by Chaleff and Stolarz (1981). This is called the booting stage, and is when the anthers in the middle of the panicle contain microspores at the late uninucleate stage.

2.1.1. Culture media

The MN6 basal medium, a modification of the N6 medium (Chu and Hill, 1988) was used for pollen embryogenesis induction. The basal medium was supplemented with 2,4-D , NAA or kinetin and 3 or 6% sucrose according to table 1.

For plant regeneration the MS medium (Murashige and Skoog, 1962), was tested also with four variations (Table 2).

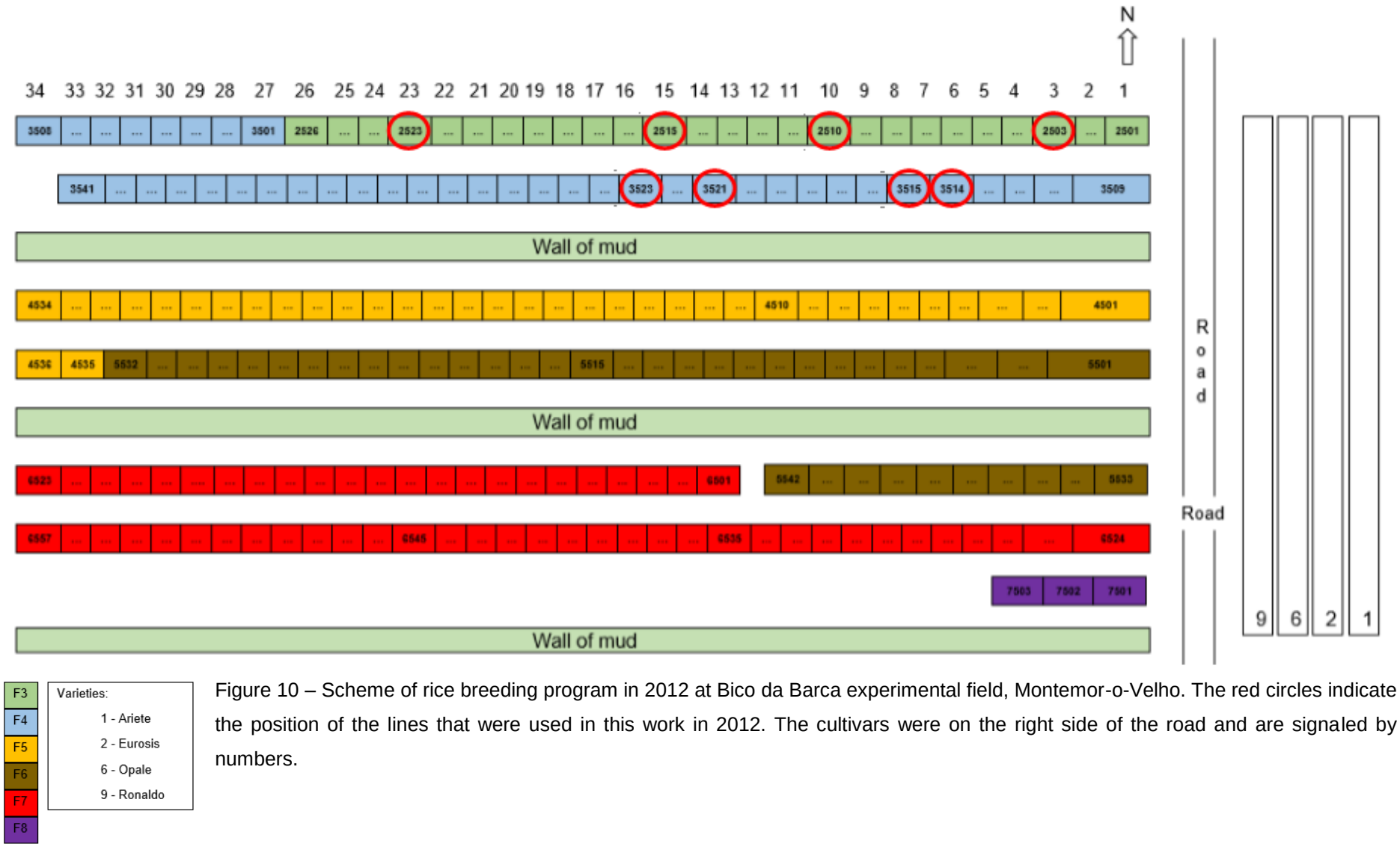

Figure 10 – Scheme of rice breeding program in 2012 at Bico da Barca experimental field, Montemor-o-Velho. The red circles indicate the position of the lines that were used in this work in 2012. The cultivars were on the right side of the road and are signaled by numbers.

Table 1 – Composition of the induction media used for anther culture.

Supplements / Medium	Basal medium	Hormones	Sucrose (%)
A1	MN6	2 mg/L 2,4-D	3
A2	MN6	2 mg/L 2,4-D 1.5 mg/L Kinetin	3
A3	MN6	1 mg/L 2,4-D 2 mg/L Kinetin 1 mg/L NAA	3
A4	MN6	2 mg/L 2,4-D	6

For callus maintenance the induced pollen callus were transferred for the same induction medium approximately 1 week after their formation. Pollen plantlets obtained in the regeneration medium were transferred to MS medium with 3% sucrose to promote further development.

Table 2 – Composition of the four regeneration media. The MS was used as basal medium.

Supplements / Medium	Basal medium	Hormones	Sucrose (%)
D3R	MS	1 mg/L BAP 0.1 mg/L NAA	3
RR1	MS	4 mg/L Kinetin 1 mg/L NAA	3
RR2	MS	2 mg/L Kinetin 0.5 mg/L NAA	3
RR3	MS	2 mg/L Kinetin 1 mg/L NAA	3

Both in the regeneration and developing media sucrose was also used as carbon source. The concentration was 3 to 6% (w/v) in the induction media (see Table 1) and 3% in regeneration media. The pH of all media was adjusted to 5.6-5.8 with HCl (0.1-1N) or KOH (0.1-1N), and 6 g/l agar (Panreac) was added. The media were sterilized by autoclaving at 120 ºC at 1.1 atm for 20 minutes.

2.1.2. Sterilization

For sterilization whole panicles were surface sterilized for 15 minutes in a filtered solution of calcium hypochlorite (7%) containing two drops of Tween 20 followed by three rinses in sterilized distilled water.

2.2. *In vitro* culture methods – Androgenesis

2.2.1. Cold-pretreatment

In 2012, some collected boots from certain varieties and lines, were wrapped and sealed in polyethylene bags and kept in the refrigerator for cold treatment at 4ºC for 10 days. The varieties were Ariete and Ronaldo, and the lines were 2523 and 2510 from the F3 generation, and 3514 and 3521 from the F4 generation. Panicles without cold-pretreatment were used as a control.

2.2.2. Anther culture

Before proceeding with anther culture, some anthers off the middle spikelets were squashed directly in a drop of acetocarmine (1%) on a slide and observed in an optical microscope to assess the stage of pollen grain development.

In a laminar flow hood, the spikelets containing microspores at the appropriate developmental stage (late uninucleate stage), were gently dissected with a sterile needle to remove the anthers which were then cultured horizontally on the media indicated in table 1. Twelve anthers were placed on each test tube and 40 tubes were used for each genotype (Figure 11). In the assays performed in 2012 only 20 test tubes were used per culture medium and

genotype. After cold pretreatment anthers were aseptically removed and cultured in the same method as the non-treated anthers. The tubes were placed in a growth chamber at 25ºC ± 1ºC, under dark conditions for 6-8 weeks, and periodically examined. After two months of culture the number of anthers showing at least one embryo or pollen callus was recorded.

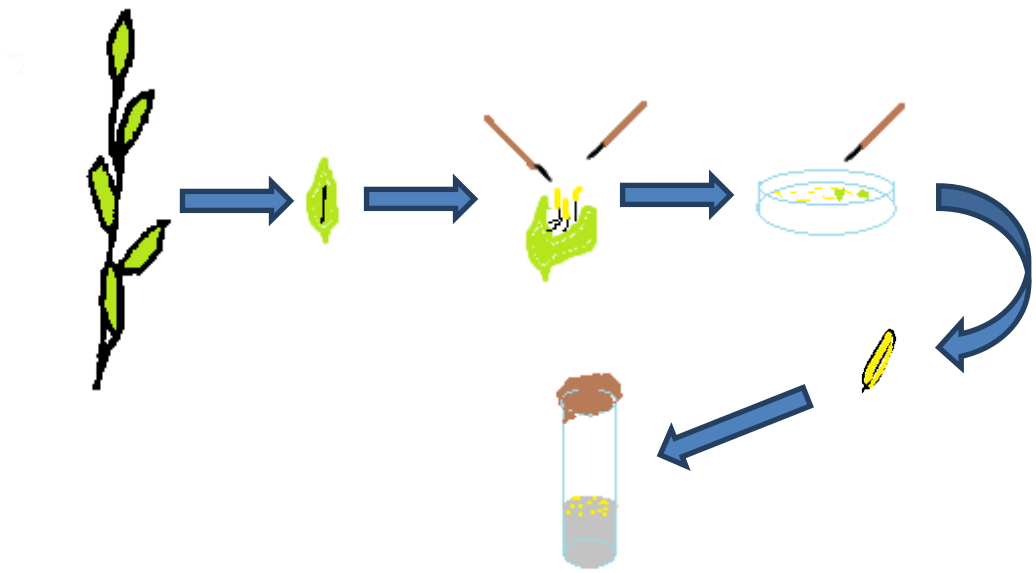

Figure 11 – Protocol adopted for rice anther culture. *T*he middle spikelets were excised in a Petri dish, in sterile conditions, and the anthers (12) placed horizontally in a test tube.

2.2.3. Plant regeneration

After calli were observed emerging from the anther, induced calli were kept 1-2 weeks in the same induction media to grow. After that period, calli were transferred to regeneration media, one callus per tube.

The tubes were incubated at 25ºC ± 1ºC, under 16 hours daily illumination with fluorescent light for plant regeneration.

Some calli were transferred to the same induction medium, and after 2-3 weeks were transferred to regeneration medium to evaluate the regeneration response after sub-culturing.

2.2.4. Plantlet growth and acclimatization

Approximately 2 weeks after calli were transferred to the regeneration media, the obtained plantlets with well-developed roots, were transferred to MS medium without growth regulators for further development. The same happened with some albino plants. However, in 2012, some plantlets were cultured again in the same regeneration medium where they have developed. The large majority of the regenerated plantlets, with well-developed root systems were transferred to pots containing autoclaved soil collected from the field where the anther donor plants were grown. The pots were placed in a growth chamber under 20 ºC, 80-90% relative humidity and a 16h light/ 8h dark photoperiod.

The large majority of the regenerated plantlets, with well-developed root systems were transferred to pots containing autoclaved soil collected from the field where the anther donor plants were grown. Plantlets were carefully removed from test tubes, gently washed to remove agar and callus debris. The roots were immersed in a solution of fungicide benlate (0.6%), to avoid contaminations and to protect the plantlet after transfer to soil. The potted plantlets were placed in a growth chamber under 20 ºC, 80-90% relative humidity and a 16h light/ 8h dark photoperiod. They were daily observed and regularly watered, with an interlude of 2 days.

2.3. Cytological studies

2.3.1. Acetocarmine squashes

This technique was used to evaluate the microspore stage before and during anther culture. On a microscope slide the anthers, dissected from the spikelet or removed from the culture tube, were squashed with a needle on a drop of acetocarmine (1%) to liberate and stain the microspores. The slides were then slightly heated on a Bunsen burner, covered with a cover slip and analyzed on a microscope (Nikon Eclipse E400), and the images were captured by a Nikon Digital Sight DS-U1 camera and observed on Act-2U software.

2.3.2 Feulgen method

The roots collected from the regenerated plants were treated with 0.05% colchicine for 2.5 h, washed with water, and subsequently fixed in acetic alcohol (1:3) over night.

The roots were then transferred to 1N HCl and incubated at 60ºC by 8 minutes in a water bath. After this hydrolysis, roots were briefly washed with water and treated with Feulgen reagent for 2-3 hours in the dark. The roots tips were placed in a microscope slide along with a drop of acetic acid 45% and squashed. The microscope slides were observed at microscope.

2.4. Flow cytometry

Flow cytometry is a technique that involves the analysis of particles optical proprieties flowing in a liquid suspension. The flow cytometer is a 5 elements system: radiation source(s), flux camera, optical filters units to select a specific wave length, photomultipliers that detect and process the interest signs and a sign processing unit (Figure 12) (Silva *et al*., 2004).

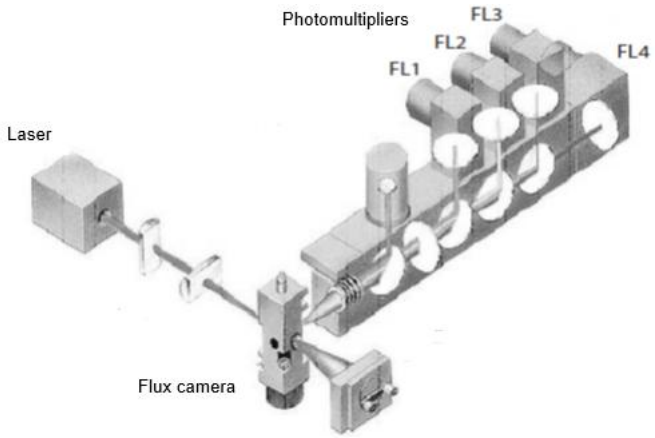

Figure 12 – Flow cytometer configuration (adapted from Silva *et al*., 2004).

The nuclei liberation technique is quite simple and fast, consisting in cut, “chopped”, small portions of leafs with a razor blade in a Petri dish containing a buffer solution, after the “chopping” the vegetal particles are filtered and

50µg/ml of propidium iodide and RNase are added (50µg/ml) to the resulting solution, then the examination in the flow cytometer takes place (Figure 13).

The data was obtained in the form of graphics of fluorescence pulse integral (FL) vs Nuclei counts, among others, through a computer software, FloMax®, that process the data in real time. The computer is connected to a flow cytometer CyFlow Space (Partec®).

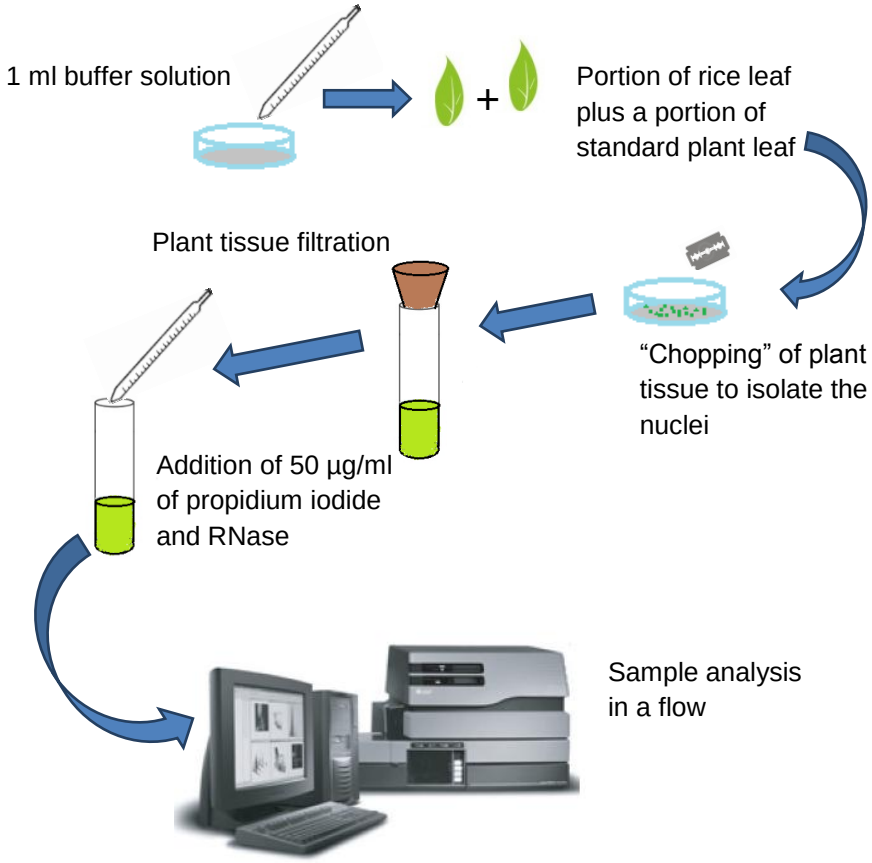

Figure 13 – Schematic diagram of the methodology used to analyze the ploidy level using plant tissue and a flow cytometer.

The ploidy level of each sample was given as a relative DNA index to the reference standard, *Pisum sativum* (DI, ratio between the sample G0/G1 peak mean and that of *P. sativum*), and for further confirmation of the ploidy level, the ratio between the G0/G1 peak of our sample and that of the control diploid standard used. Since two plant material were analyzed simultaneously, histograms generate two G1 pikes, the first one relative to samples of rice plants, while the second one represents the standard plant.

The ploidy genome size in pg of our samples was estimated by multiplying the DI by 9.09 pg (the genome size of the reference standard, Pisum sativum). Knowing that *O. sativa* have a small genome of approximately 2C = 1.00 pg of DNA content, the ploidy of the regenerated plants was obtained.

2.5. Statistical analysis

Statistical analysis was performed with Statistica 7 for Windows®, employing a significance level (α) of 0.05. The evaluation of the effect of induction media was performed by One-way ANOVA, after a square root data transformation. For assessment of the cold-pretreatment on each line and variety and the induction medium was tested by using a t-student test.

3. Results

Results

3.1. Androgenesis induction

3.1.1. Effect of the microspore stage of development

Squashes in acetocarmine before anther culture have shown that most of the microspores were at the uninucleate vacuolated stage, usually known as mid-uninucleate stage. The uninucleate microspores showed a central large vacuole and a peripheral nucleus indicating the proximity of the first pollen mitosis, (Figs. 14A-B). However, some smaller microspores containing starch grains were also observed (Fig. 14C). Cytological observations of the microspores were also performed after the cold-pretreatment showing the presence of late-uninucleate microspores and a large number of plasmolyzed microspores (Fig. 14D). The stage of the microspores varied depending on the panicle location.

Within 3-4 days of culture the smaller grains degenerated, and only the bigger microspores were viable and started to divide, presenting two nuclei approximately of the same size (Fig. 14E). After 10-15 days of culture, cell division rate increased, resulting in the formation of multicellular pollen (Fig. 14F), though some microspores showed a certain degree of plasmolysis.

3.1.2. Callus formation and development

The continued cell division eventually caused the rupture of the exine and, about 2-3 weeks after the anther culture, unorganized calli were formed emerging from within the anther (Fig. 15A). Sometimes, more than one callus per anther were produced, but at later stages they merged to form a unique callus mass in which calli of different origins were difficult to distinguish (Fig. 15B).

On these calli nodular calli embryoids at the globular stage of development appeared. At the same time, the anther tissues have turned brown indicating their senescence (Fig. 15C). After 4-5 weeks of anther culture the calli reached

a considerable size (3-4 mm), and were transferred to the regeneration media (Fig. 15D).

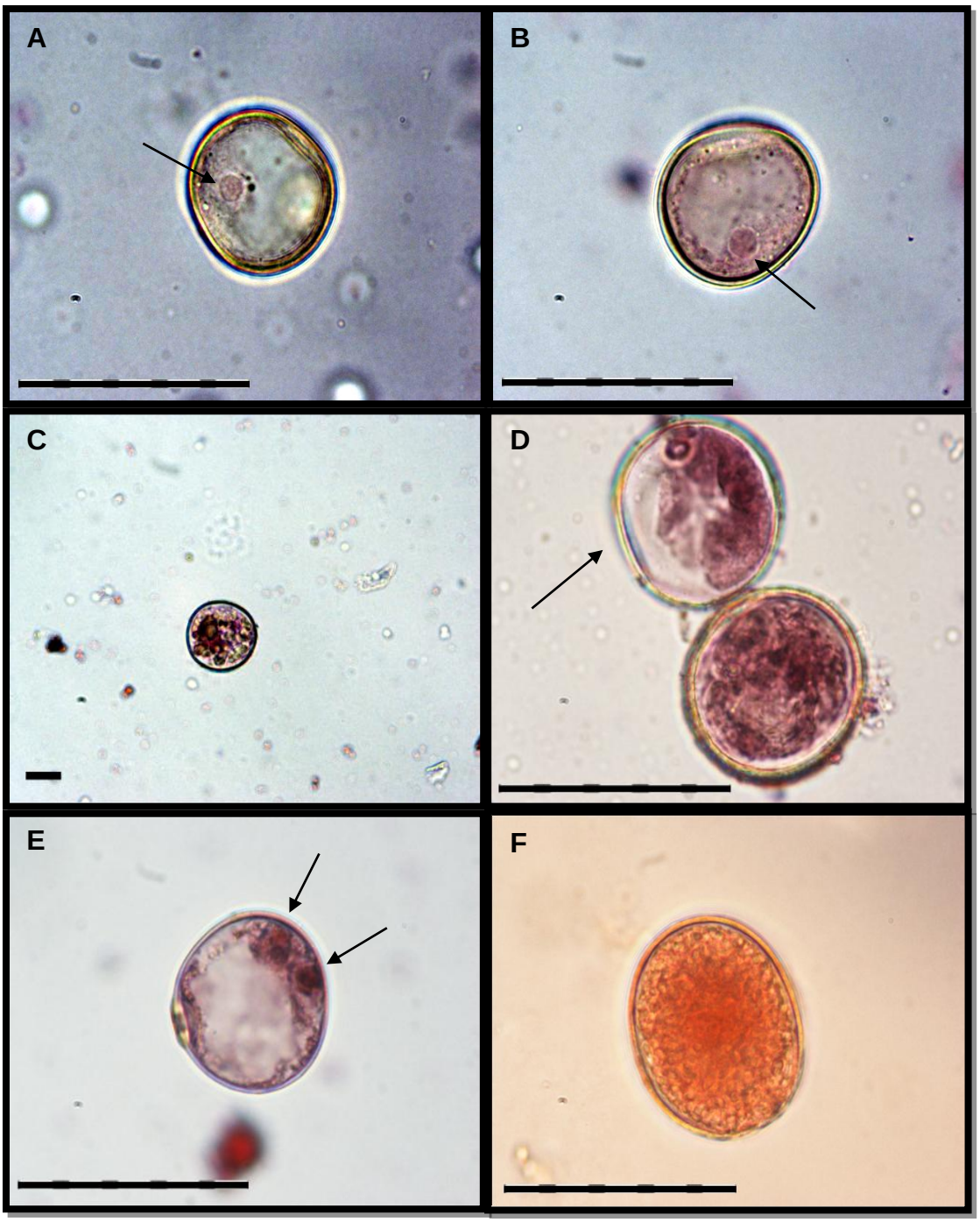

Figure 14 – Pollen behavior during anther culture. A and B: uninucleate microspores showing a large central vacuole and a peripheral nucleus (arrow) at the time of the anther culture; C: small microspore with starch grains which latter degenerate; D: partial plasmolyzed microspore (indicated by the arrow); E: binucleate microspore where a large vacuole can be seen (arrows indicate the nuclei); F: multicellular pollen. Bars = 50µm.

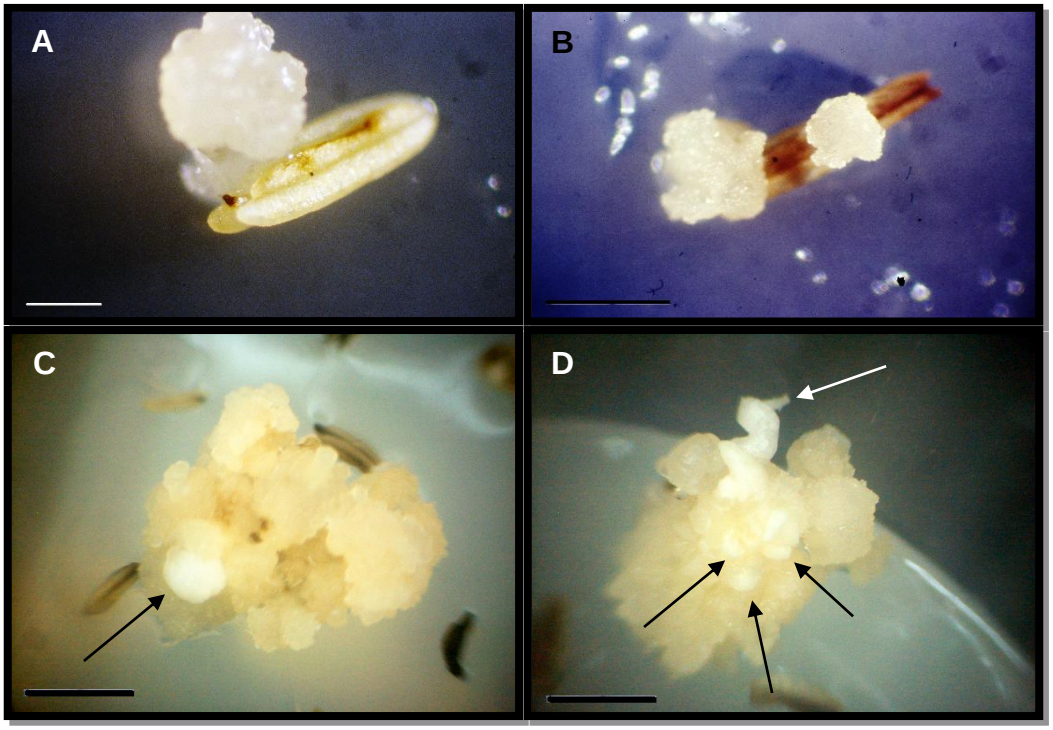

Figure 15 – Callus formation and development in rice anther cultures. A: Callus emerging from inside the anther through the dehiscence lines; B: two microcalli formed in the same anther; C: callus in continued cell division showing an embryo at the globular stage (arrow); D: well-developed callus with globular embryos (black arrows), and one more advanced embryo (white arrow). Bars = 2mm

3.2. Induction results

Assays carried out in 2010:

In 2010 the effect of the induction media on the induction rate was tested.

The results showing the frequency of callus induction and plantlet regeneration are presented in table 3. For each induction medium about 2400 anthers were cultured per line/cultivar. The regenerated plants are represented in the same tables as the induction frequency since none of the regenerated plantlets survived when transferred for pots with a substrate different from that found in rice culture fields.

Table 3 – Callus induction frequency and number of regenerated plantlets on the induction media in 2010. Callus formation (%) was obtained by the ratio of anthers that produce callus and the total number of cultured anthers.

Induction Medium	Line/Variety	Callus formation (%)	Number of Regenerated plantlets	
			Green	Albino
A1	2501	1.25	0	0
	2503	4.17	4	5
	2504	3.75	4	2
	2506	1.67	3	0
	2531	0.83	0	0
	2536	0.00	0	0
	Eurosis	0.42	0	0
	Ariete	0.00	0	0
	Dardo	4.17	0	0
	Sirio	1.67	0	0
A2	2501	0.00	0	0
	2503	4.17	2	1
	2504	2.92	0	0
	2506	0.00	0	0
	2531	0.00	0	0
	2536	0.00	0	0
	Eurosis	0.42	0	0
	Ariete	0.00	0	0
	Dardo	0.00	0	0
	Sirio	0.00	0	0
A3	2501	10.0	4	13
	2503	4.17	2	0
	2504	4.58	1	1
	2506	2.08	0	0
	2531	12.9	1	2
	2536	0.83	1	0
	Eurosis	3.75	0	0
	Ariete	0.42	0	0
	Dardo	4.17	1	6
	Sirio	0.83	1	2
A4	2501	0.42	0	0
	2503	5.00	5	6
	2504	2.92	6	10
	2506	1.25	0	6
	2531	1.67	0	0
	2536	0.00	0	0
	Eurosis	0.00	0	0
	Ariete	0.83	0	0
	Dardo	1.25	0	0
	Sirio	0.00	0	0

Taken together, the results of this assay showed that callus formation in all the lines/cultivars tested was better on the A3 medium (4.38%) followed by the A1 medium with an induction rate of 1.79%. The A2 and A4 media produced the lowest responses with 0.75% and 1.33%, respectively (Fig. 16). Callus induction was significantly affected by the induction media (p= 0.003561), with A3 medium callus induction being statistically different from the A2 medium. The number of regenerated green plantlets obtained was the same for all induction media (11 plantlets) except in A2 in which only 2 green plantlets were formed.

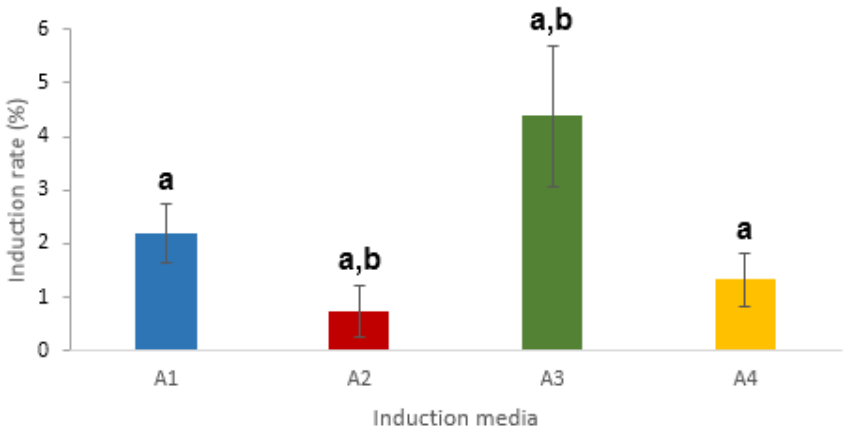

Figure 16 – Induction response on the four different induction media. Bars followed by different letters were significantly different.

When comparing the breeding lines and cultivars it can be seen that cultivars gave, in general, better results with the line 2503 presenting the best induction rate. Nevertheless, the lowest induction rate was obtained with line 2536. Among cultivars, Dardo was the most responsive (Fig. 17).

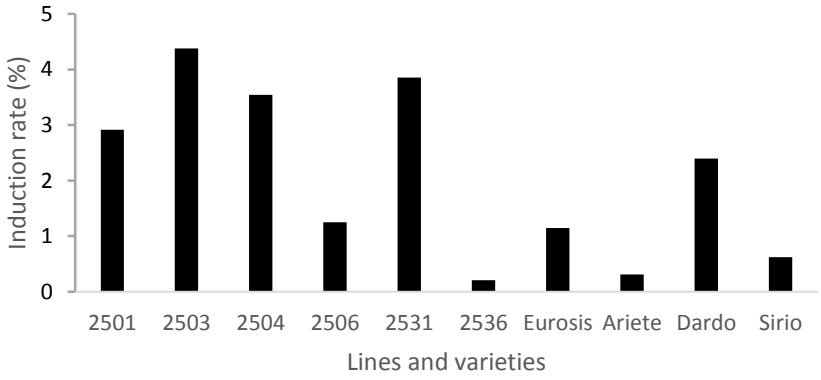

Figure 17 – Pollen callus formation in different lines and cultivars tested in 2010. Results obtained after 2 months of culture.

Assays performed in 2011:

In 2011, six more lines and four cultivars were tested. However, in this assay, only the most two responsive medium tested in 2010 were used (A1 and A3). Table 5 shows the induction rates obtained in the assay.

Table 5 - Callus induction frequency on the A1 and A3 induction medium in 2011. Callus formation (%) was obtained by the ratio of anthers that produce callus and the total number of cultured anthers.

Induction Medium	Line/cultivar	Callus formation (%)
A1	1131	0.00
	1133	11.25
	1134	6.47
	2520	2.08
	2524	5.63
	2527	1.66
	Ariete	5.00
	Eurosis	1.04
	Opale	1.88
	Ronaldo	2.71
A3	1131	0.00
	1133	9.58
	1134	7.71
	2520	8.96
	2524	3.96
	2527	5.63
	Ariete	3.54
	Eurosis	5.21
	Opale	4.38
	Ronaldo	5.42

The results were in line with the 2010 experiments, showing that A3 medium was the best for androgenesis induction, with a mean induction rate of 5.44%, a value slightly higher than the result of 2010. F2 line 1133 was the most responsive line on both media. The line giving the worst results was the

F2 line 1131 in which callus formation could not be achieved. In the cultivars Eurosis and Opale pollen embryogenesis was reduced (Fig. 18).

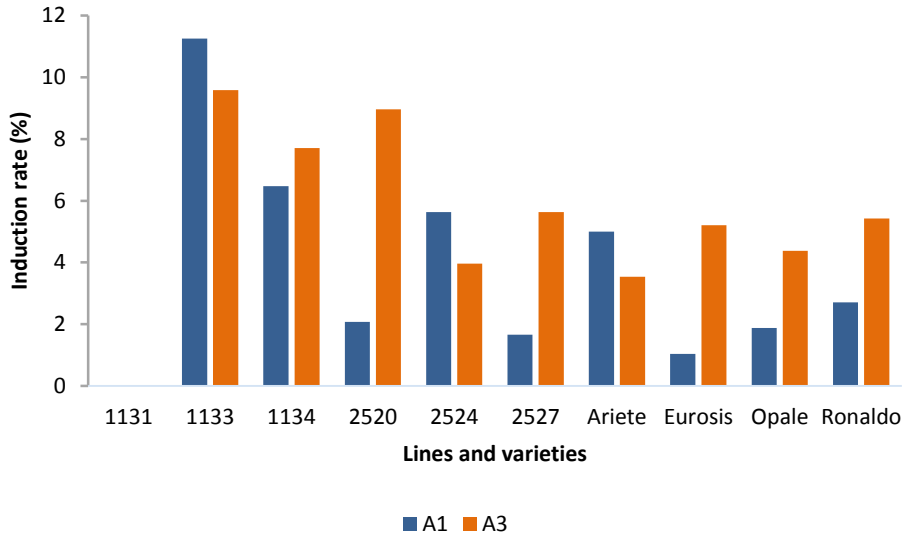

Figure 18 – Response of different cultivars and breeding lines on media A1 and A3. Results obtained after 2 months of culture.

Data analysis indicate that F2 lines (1133 and 1134) usually gave better results than the F3 (2524 and 2527) lines. However, all the tested breeding lines were able to undergo androgenesis. Calli transferred to the regeneration media were unable to produce plants in any of the media and lines tested.

Assays carried out in 2012:

Five of the lines used in 2012 were descendants of lines used in the year before: line 2523 F3, was the F2 line 1134; the F4 lines 3514 and 3515, 3521 and 3523, were the F3 lines 2520 and 2527, respectively.

Table 6 - Callus induction in different lines and cultivars cultured in 2012, on A1 and A3 medium. Callus formation (%) was obtained by the ratio of anthers that produce callus and the total number of cultured anthers.

Induction Medium	Line/cultivar	Callus formation (%)
A1	2503	0.00
	2510	0.63
	2515	0.21
	2523	4.58
	3514	0.21
	3515	1.46
	3521	4.79
	3523	2.29
	Ariete	0.00
	Eurosis	1.25
	Ronaldo	0.00
	Opale	0.21
A3	2503	0.21
	2510	0.83
	2515	0.63
	2523	2.08
	3514	0.63
	3515	12.71
	3521	3.10
	3523	1.04
	Ariete	0.00
	Eurosis	2.50
	Ronaldo	0.00
	Opale	1.04

Table 6 shows that cultivars Ariete and Ronaldo did not produce androgenic calli, which is in marked contrast with the results obtained in 2011. Eurosis and Opale were the only cultivars to respond on both media, with Eurosis induction being slightly higher than in Opale (Figure 19).

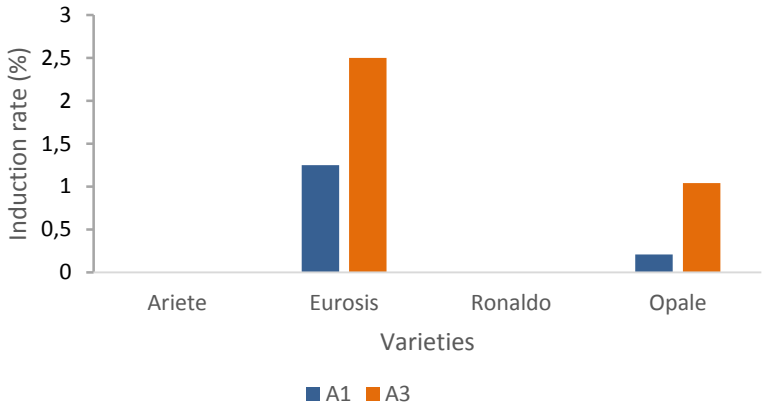

Figure 19 – Induction rate in the cultivars used in 2012. Results obtained after 2 months of culture.

Regarding the response of breeding lines, it was obvious that the F4 lines have a better androgenic response compared to F3 lines, with 3515 line exhibiting a high induction rate on A3 medium (12.71%), higher than last's year when was the F2 line 2520. The exception is line 3514, with the same parent line, but presenting a very low response on both media. Lines 3521 and 3523, previous 2527, had similar results between 2011 and 2012 with a slightly decrease on the last one. The F3 lines, which in 2011 showed an interesting behavior as F2 lines, showed a pronounced decrease. For example, the 2523 line which as F2 line 1134 gave the highest frequencies of induction during 2011 suffered a huge reduction of induction, even though still represents the best results between their homologous, since the remain F2 lines had a very low induction rate (Figure 20).

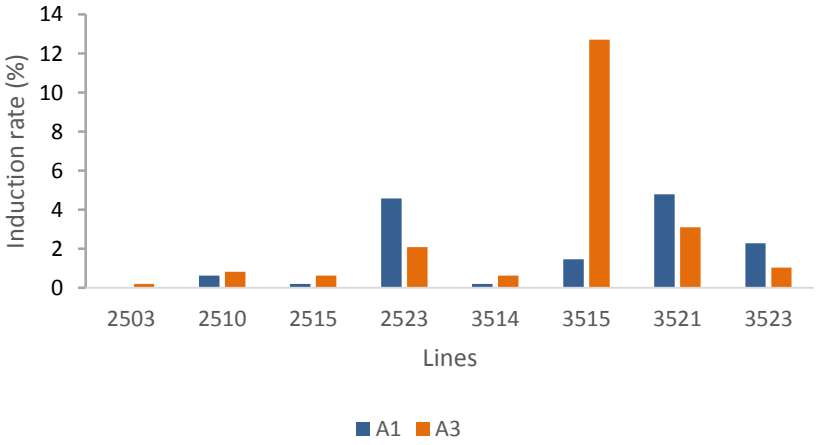

Figure 20 – Comparison of the induction frequencies between F2 and F3 lines used in 2012.

3.2.1. Effects of sucrose and plant growth regulators

Sucrose and plant growth regulators (PGRs) were tested on the pollen embryogenesis induction. On the induction media used on this work, two sucrose concentrations were used: 3% and 6%, the last one only on A4 medium. Three growth hormones were used in different concentrations, 2,4-D, NAA and kinetin.

The results showed that A3 medium, that had the three growth hormones, gave the best induction response, with statistical differences when compared to A2 medium that gave the lowest response.

On the regeneration media sucrose concentration was the same for the four media, 3%, however growth hormones and their concentration had some variations. To induce plant regeneration the auxin NAA and cytokinins BAP (6-Benzylaminopurine) and kinetin were used. BAP was only used on D3R regeneration medium at 1 mg/L. whereas NAA was tested at different concentrations (0.1 mg/L; 0.5 mg/L; 1 mg/L), as well as kinetin (2 mg/L; 4 mg/L). Plantlet regeneration were mainly obtained on RR1 medium, supplemented with 4 mg/L of kinetin and 1 mg/L of NAA.

3.3. Cold-pretreatment effect on androgenesis induction

In 2012, the effect of cold-pretreatment of 10 days at 4ºC on androgenesis induction was tested on some lines and cultivars.

On A1 medium, the cultivars used, Ariete and Ronaldo gave different responses: the first one did not produced calli on control neither after cold shock, whereas Ronaldo do not produced any callus on control conditions as well, but after cold shock some calli were induced. Among F3 lines clear differences could be seen. Line 2510 that had less than 0.1% induction response on control, gave more than 0.5% callus induction after cold-pretreatment. By contrast the second F3 line tested, 2523, had a higher response without the cold-pretreatment, with only 0.05% induction success on treated anthers. F4 lines displayed similar results, with line 3514 having the higher induction rate on A1 medium after cold-pretreatment (0.725%), and line

3521 lacking response on cold-treated anthers opposed to the control where a rate of 0.575% occurs (Figure 21).

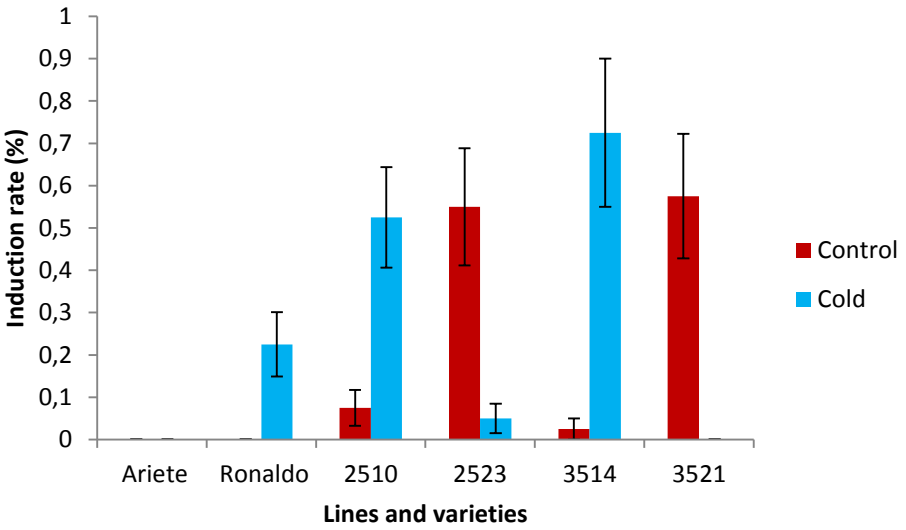

Figure 21 – Callus induction on A1 medium with and without cold-pretreatment. Values are the mean ± SE. Results obtained after 2 months on the induction medium.

Testing the cold-pretreatment effect on A1 medium with a t-student test ($\alpha = 0.05$), statistical differences were found on every line and cultivar, although line 3514 presented the higher differences between control and cold-pretreatment ($p=0.000169$).

In general, identical results could be found on A3 medium. Ariete cultivar did not respond both in control and with cold shock, and Ronaldo only respond after the cold-pretreatment. Among F3 lines, 2510 presented the higher induction rate after cold-pretreated, with 1.13% of callus formation, and in 2523, control had again a higher response when compared with cold-pretreatment. In what concerns F4 lines, 3514 had a better induction rate after the cold shock; however, lower than in A1 medium, and finally line 3521, that do not respond to cold-pretreatment on A1 medium, had a 0.35% induction rate on A3 medium, however slightly lower than the control (0.375%, Fig. 22).

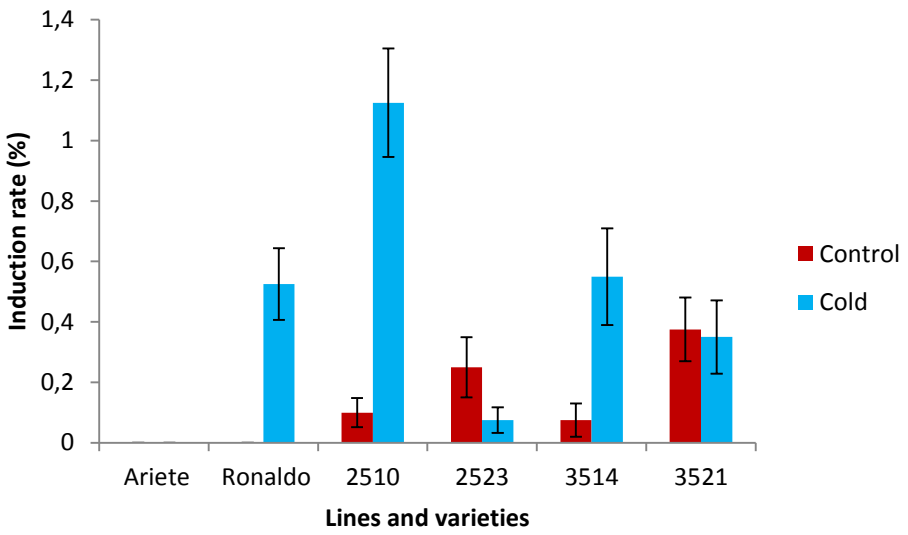

Figure 22 - Callus induction on A3 medium with and without cold-pretreatment. Values are the mean ± SE. Results obtained after 2 months on the induction medium.

After statistical analysis on the results on A3 medium, statistical differences could not be found between lines 2523 and 3521, nonetheless clear statistical differences among the remain lines and in Ronaldo were found, with line 2510 presenting the higher statistical difference ($p<0.01$).

3.4. Plant regeneration

In 2010, D3R medium allowed the regeneration of 35 green plants and 54 albinos. However none of the green plants survived acclimatization, in a different soil than the one used in 2012. In 2011, not a single plant was obtained when the same medium was used.

In 2012, the regeneration process was different, and four regeneration media were tested: the same D3R medium and three new media, RR1, RR2 and RR3. The number of calli placed on each regeneration media following induction is shown in table 7. A total of 343 plantlets were regenerated on the four media.

Table 7 – Number of calli placed per regeneration medium following induction on A1 or A3 media.

Regeneration medium	Number of callus		Regenerated plants	
	Induction medium			
	A1	A3	A1	A3
RR1	35	36	36	202
RR2	34	34	56	1
RR3	27	44	0	22
D3R	31	43	0	26

Well-developed calli were placed on the regeneration media, and the embryos started to develop evolving into plantlets showing well developed shoots and roots (Figs. 23A-B). Although plantlet regeneration had been obtained in all media, some calli initially regenerated roots, and none of these calli that regenerated roots prior to shoots would differentiate into plants, dying afterwards (Fig. 23C). On the contrary, calli that initiated shoots would eventually develop into complete plants, with roots appearing after shoot development (Fig. 23D). More than one plant could be usually regenerated per callus, and sometimes albino plants and green plants developed on the same callus (Fig. 23E).

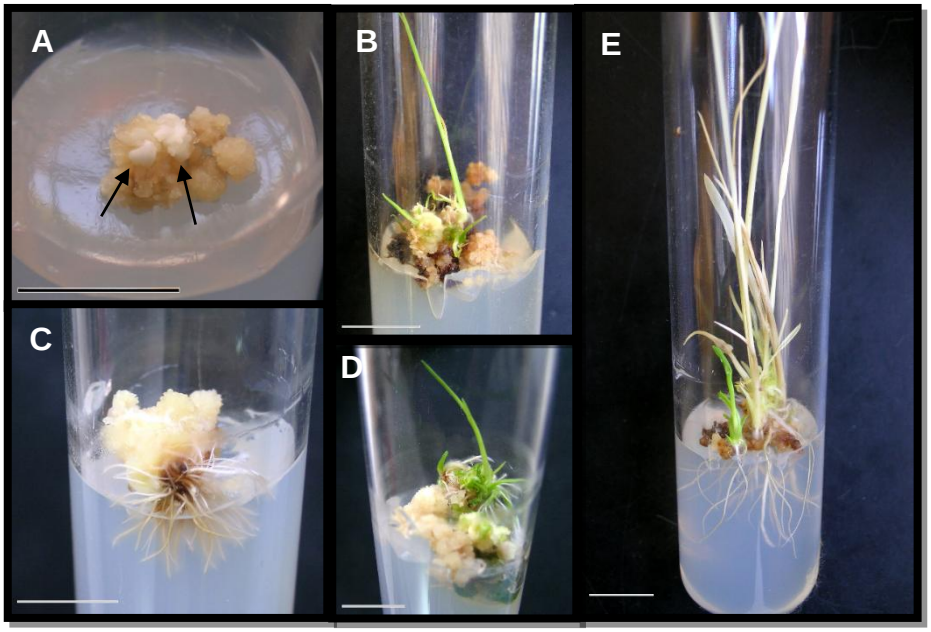

Figure 23 – Plantlet formation. A: well-developed callus with embryos (indicated by the arrows) on regeneration medium. B: differentiation of the callus with formation of shoots. C: Root formation. D: callus that regenerate shoots before roots and then started to form roots along the process. E: regeneration of green and albino plantlets on the same callus. Bar = 1cm.

The number of calli was similar in the four regeneration media, with 74 tubes (calli) of D3R, 71 of RR1, 68 of RR2 and 71 of RR3. Nevertheless, the number of plantlets obtained was considerably different for all the four media. Not all the lines and varieties that succeeded on forming callus could regenerate plants, and only four lines and one cultivar regenerated plantlets. Eurosis was the only cultivar that regenerated plants.

Among the breeding lines, F4 lines 3523, 3515 and 3521 and the F3 line 2510 were able to produce plantlets, though all the plantlets of line 2510 and a part of 3521 plantlets resulted from the calli of cold-pretreated panicles, named from now on 2510F and 3521F.

The number of culture tubes per line/cultivar were approximately the same, yet the regeneration success is quite different in breeding lines and Eurosis. Thus, line 2510F presented the highest number of regenerated plantlets (238 plantlets), whereas in the cultivar Eurosis only 13 plantlets were obtained (Figure 24).

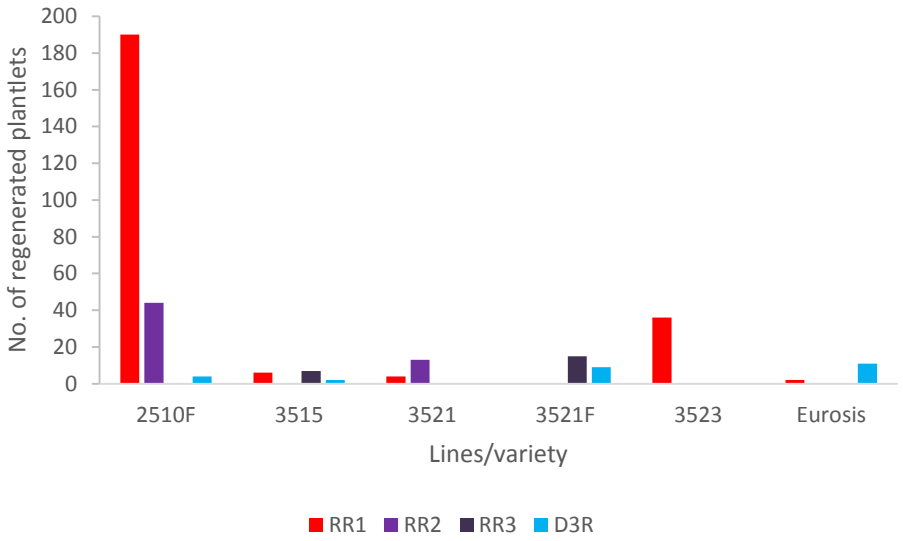

Figure 24 – Number of regenerated plantlets in breeding lines and cultivar Eurosis. Results recorded after 2 weeks in the regeneration media.

The figure above clearly shows that neither the breeding lines or Eurosis, were able to form plantlets on all four regeneration media, and only lines 2510F and 3515 regenerated on three of the four media, with line 2510F being the most responsive, especially when RR1 medium was used. Comparing line 3521 with and without cold-pretreatment, it can be seen that there is no considerable differences on the number of regenerated plantlets, with 3521F only 7 plantlets over than control 3521 (Fig. 24).

RR1 medium presented the best results (Fig. 25), however, a great part of the regenerated plantlets on this line and medium were obtained by placing recent regenerated plantlets on the same regeneration medium. This procedure allowed the formation of shoots around the inoculated plantlet (Figs. 26A-B).

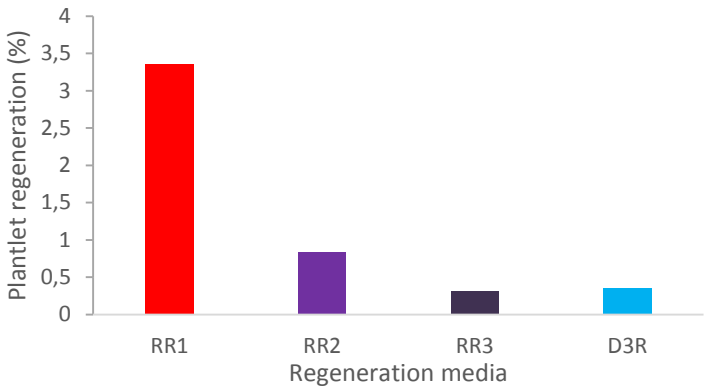

Figure 25 – Plantlet regeneration in different media. Results obtained after approximately 2 weeks of culture.

RR1 medium presented the best results per culture tube, with more than 200 plantlets regenerated in total; on the other side RR3 and D3R medium gave the lowest responses.

Albino plant formation was a common feature of this work (Fig. 26C). In 2010, a total of 54 albino plantlets were regenerated, especially in the 2501 and 2504 lines. In 2012, despite the high number of green plantlets achieved, 151 albino plantlets were formed during the regeneration process, with RR3 medium being the medium in which the highest rate of regenerated albino plantlets was recorded (Fig. 27).

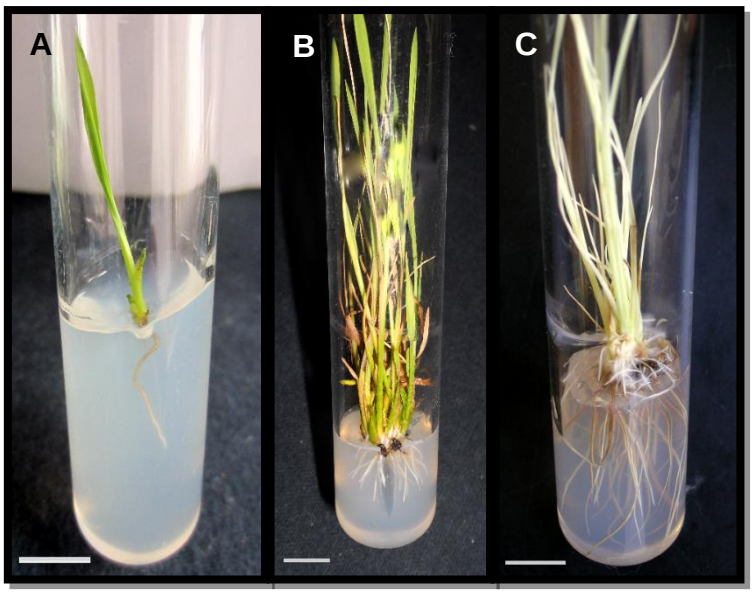

Figure 26 – Plantlet formation. A-B: regenerated green plantlets placed again on regeneration medium and the consequent formation of various plantlets through time. C: regeneration of albino plantlets. Bar = 1cm.

Among the lines, 2510F presented the highest number of albino plantlets (64). The cultivar Opale and line 3514F did not regenerate any green plantlet but had the formation of 3 albinos each. In Eurosis no albino plantlets appeared (table 8).

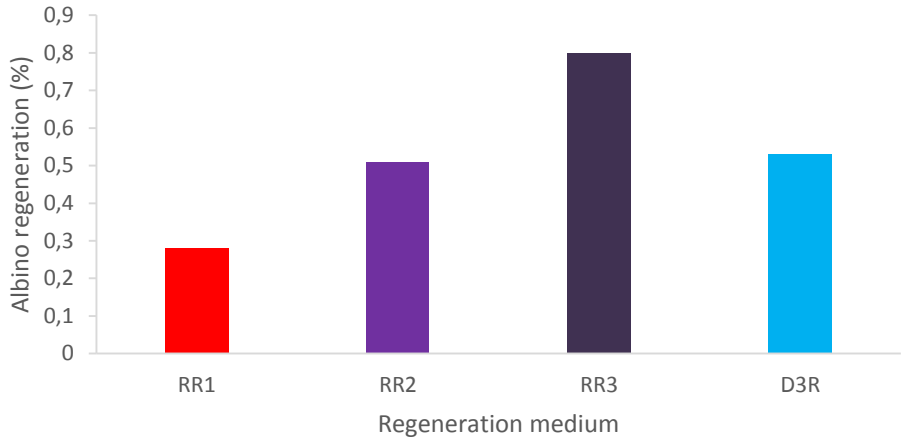

Figure 27 – Albino plantlet formation in different regeneration media.

RR3 medium was the more prone to albino plantlet formation whereas RR1 gave the lowest frequencies. This results reinforce the idea that RR1 medium is more appropriate for plantlet regeneration because not only gave the best frequencies of regeneration but also the lower rate of albino formation.

Table 8 – Number of albino plantlets regenerated in the cultivars and breeding lines tested

Lines/cultivars	N0. of albino plantlets regenerated
2510	1
2510F	64
3514F	3
3515	17
3521	25
3521F	20
3523	18
Eurosis	0
Opale	3

3.5. Flow cytometry

After complete acclimatization of the plants, those who survived were analyzed by flow cytometry to determine ploidy level. Using common pea (*Pisum sativum* L.) as the reference plant of known genome size (2C = 9.09 pg), the DNA content assessment was made through the relative position of G0/G1 pike of the analyzed plant compared to the reference standard plant G0/G1 pike position.

The flow cytometry histograms usually generate G0/G1 and G2 pikes, that indicates, respectively, that the major part of the nuclei are in G0/G1 phase of interphase and those who are in G2 phase. The G2 pike is not always detected. The interlude between the two pikes represents the DNA synthesis period, the S phase.

Over the 114 plants analyzed, haploid, diploid and triploid plants were obtained (Fig. 28).

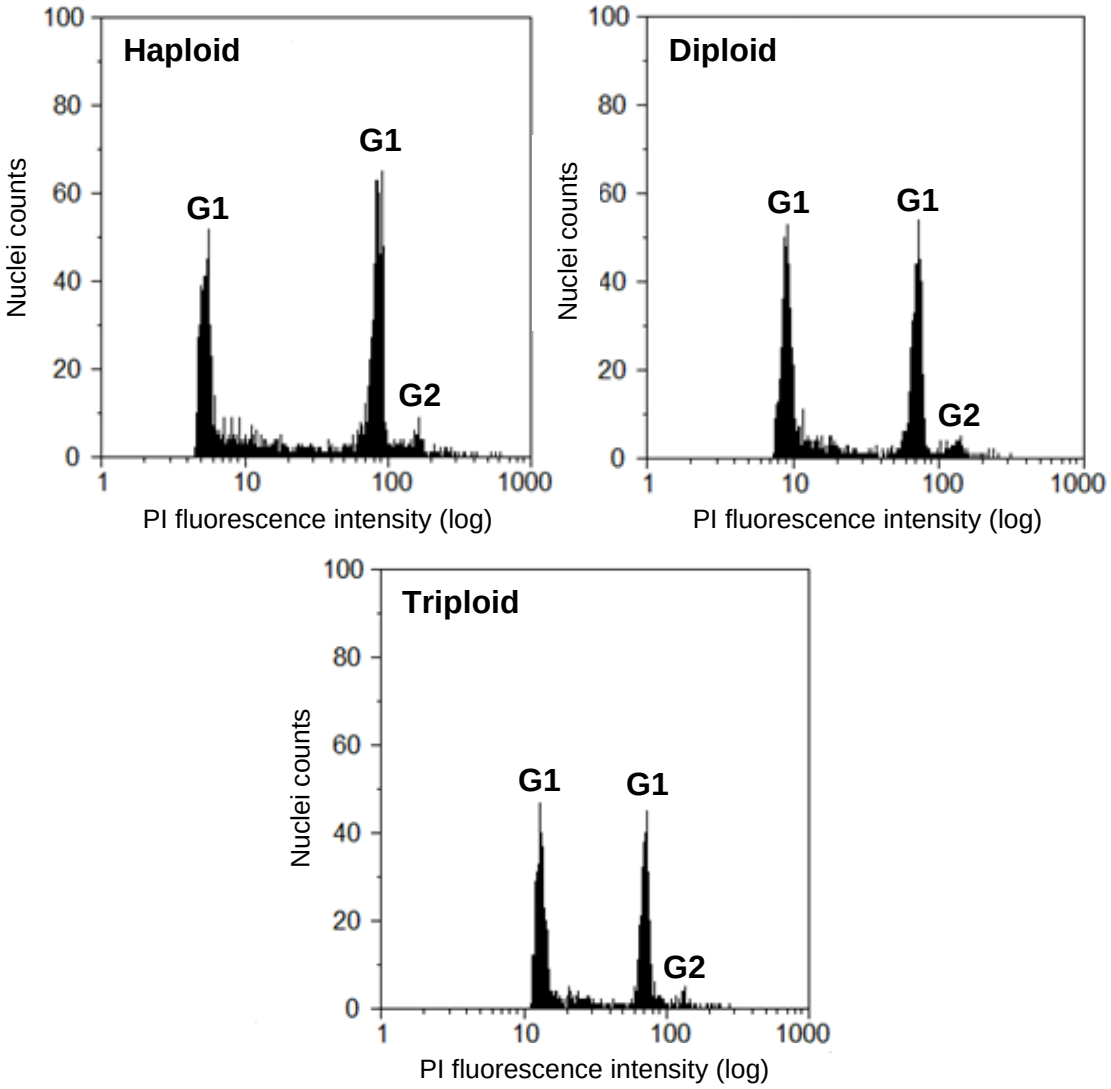

Figure 28 - Flow cytometry histograms of three different lines of regenerated plants. The left G1 pike refers to the regenerated rice plant sample and the second one to the standard plant. G2 pike is not perceptive on rice samples. Among the regenerated plants, ploidy varies from haploid to triploid. Haploid: line 2510; Diploid: line 3523; Triploid: line 3521.

From the 114 regenerated plants analyzed the haploid condition was the most common (77.19%). Line 2510 with cold-pretreatment represent most of the haploid plants (95.46%), with line 3515 and Eurosis the other haploid plants source (Figure 29).

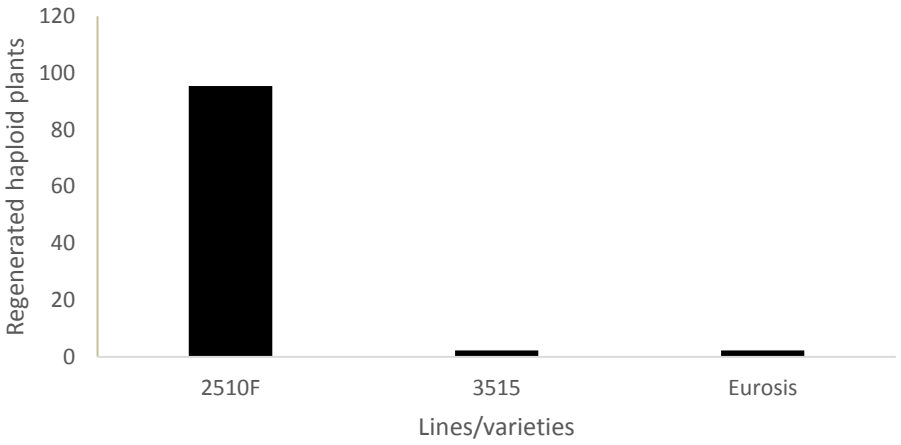

Figure 29 – Haploid plants regenerated by cultivar/line.

Among diploid plants F4 lines, 3523 and 3521, gave the highest number of plants regenerated (Figure 30). All the lines and Eurosis regenerated diploid plants (20.18%), and although spontaneous chromosome doubling is frequently observed during callus development in rice haploids, the possibility that these diploid plants were initiated from the somatic cells of the anthers could not be entirely excluded.

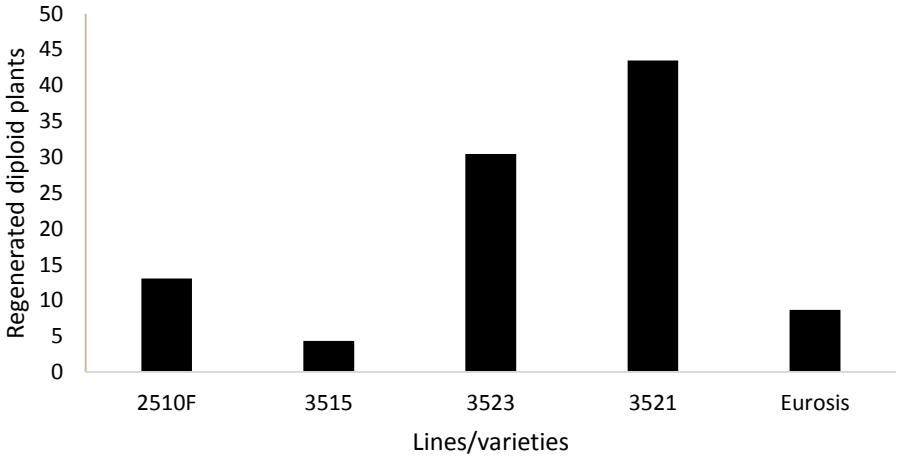

Figure 30 – Diploid plants regenerated by cultivar/line.

Triploid plants were also found among the regenerated plants: 3 plants, all from line 3521 following cold-treatment. This F4 line gave the highest rate of diploid plants as well, and did not regenerated any haploid plant.

Some calli were also analyzed by flow cytometry after two sub-cultures showing tetraploid and mixoploid conditions (Fig. 31).

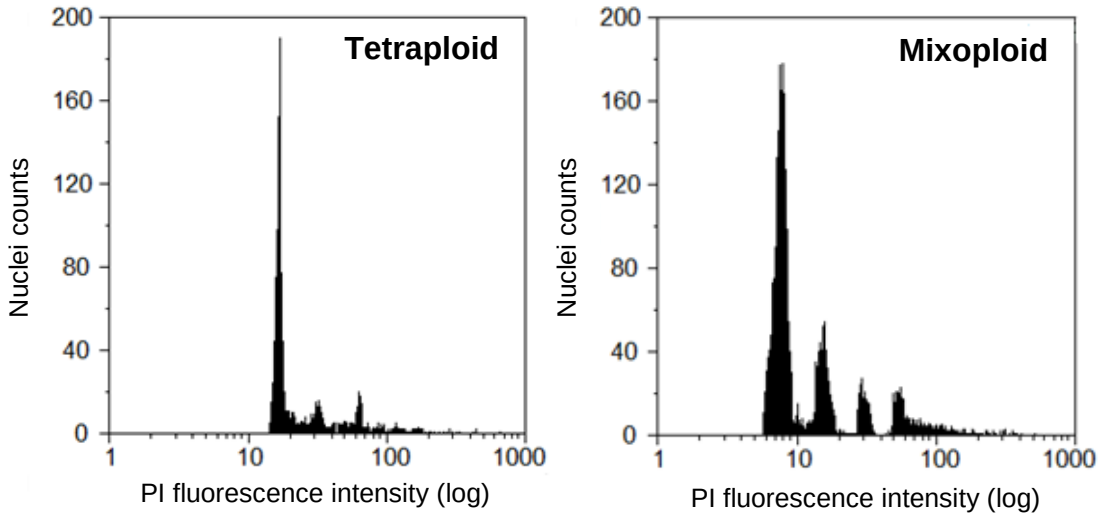

Figure 31 – Flow cytometry histograms of two different callus. Tetraploid: callus from line 2523. Mixoploid: callus from variety Eurosis.

Over the three years of work it became clear that, after two sub-cultures, callus lose their regeneration capacity, and not a single plant was obtained after that moment. The histograms of figure 31 clearly show what happens in sub-cultures, with calli changing their ploidy status rapidly.

A ploidy evaluation was also made to an albino plant, showing a diploid condition.

3.6. Chromosome counting using the Feulgen technique

In this work chromosome counting was tried in root tips of the regenerated plantlets. However, rice chromosomes are very small and have the tendency to aggregate which makes difficult their observation and counting.

Nevertheless, some microscope observations at 1000x magnification allowed the visualization of chromosomes in some root tips of haploid plants. Although the exact number of chromosomes could not be counted, observations of a few samples seem to indicate they have only half the set chromosomes (n = 12, Figure 32).

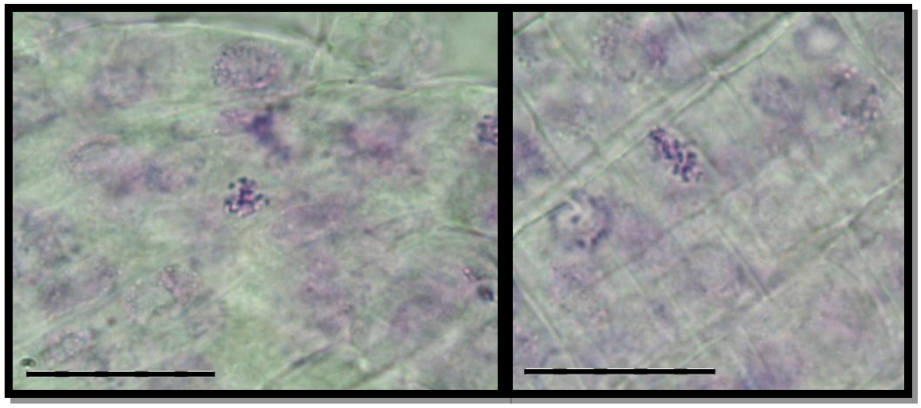

Figure 32 – Haploid chromosome set in root tips, 12 chromosomes. Aggregation is visible on both images, and counting was very difficult to make. Bar = 50µm

3.7. Plant acclimatization and flowering

The regenerated plantlets were placed on a suitable substrate in a growth chamber Aralab, with controlled conditions. From the 343 regenerated plantlets only 114 survived to acclimatization (Fig. 33). The first week is critical for the acclimatization, and then constant careful with watering is necessary, with the rice plants being in constant contact with water simulating the field conditions.

About 2-3 months the plants had grown from a small plant to considerable size (Figs. 34A-B), and started to flower (Fig. 34C).

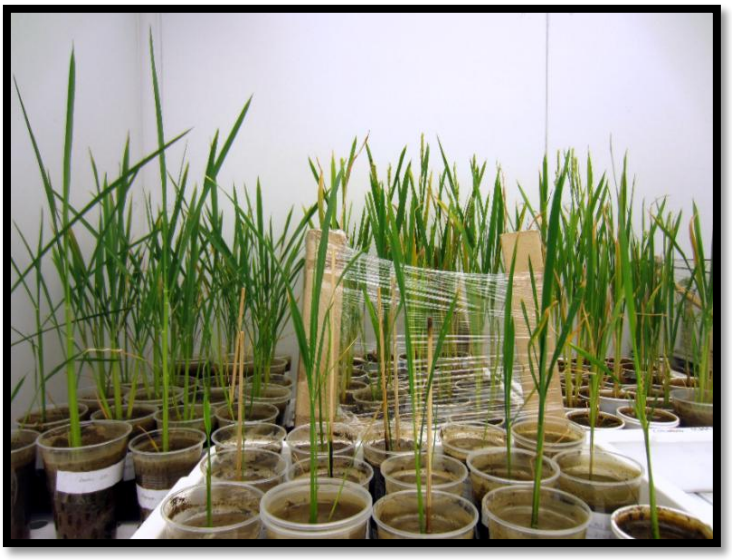

Figure 33 – Acclimatized plants on controlled conditions. The recent potted plants are on the front sector of the figure, smaller and still very fragile, then the older ones, already with a considerable size are visible in the back.

Two days watering is needed to maintain the water level on the cups.

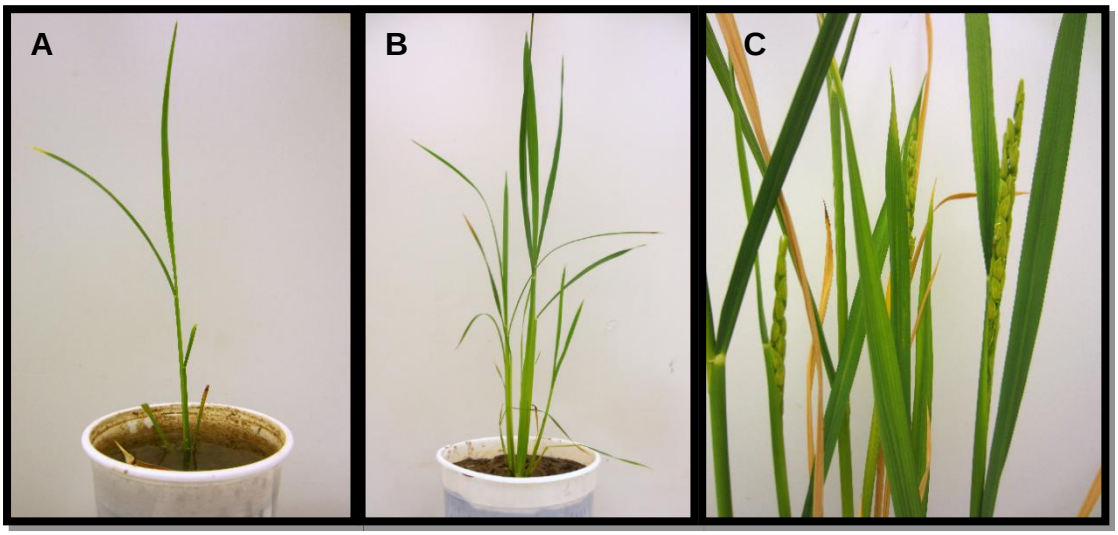

Figure 34 – Plant maturation in growth chamber. A – one-week old plantlet in controlled conditions. B – one-month old plant, with tillers. C – spikelets formed in plant approximately after three months in substrate.

3.8. Phenotypic differences between haploid and diploid plants

After ploidy evaluation by flow cytometry, phenotypic characteristics were observed in haploid and diploid plants. Although both haploid and diploid plants produced flowers, there are clear distinct features between them.

The first obvious difference among the plants is the size: haploid plants were clearly smaller, and sometimes presented more shoots than diploid plants (Figure 35).

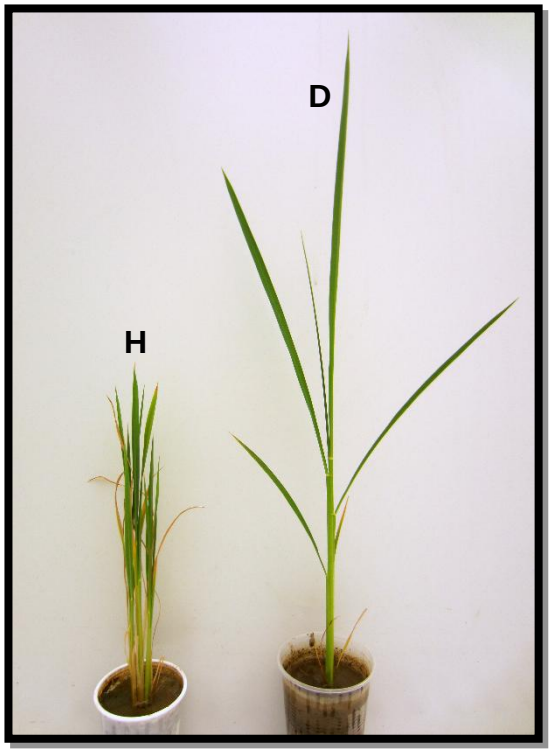

Figure 35 – Size and plant architecture differences between haploid (H) and diploid (D) plants.

Although the plants on the figure above were placed in acclimatization at the same time the size differences are clear.

Triploid plants had approximately the same size of diploid plants with no clear distinct differences between them.

Among the haploid and diploid plants the differences are quite obvious, and the observation of the sexual structures confirmed the differences.

Haploid spikelets are smaller than diploid ones, and sexual structures are clearly smaller and atrophied, and both male and female structures were different on diploid and haploid plants (Fig. 36).

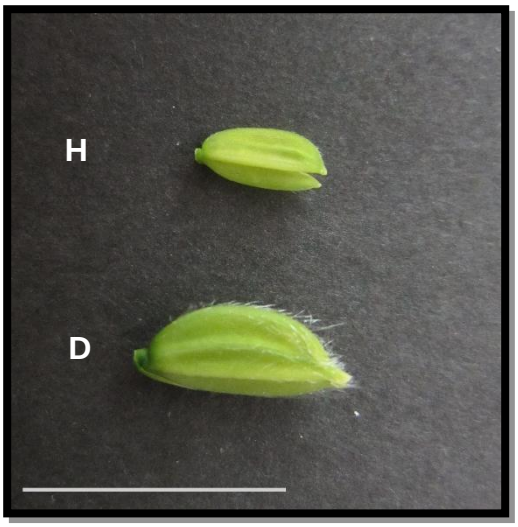

Figure 36 – Haploid (H) and diploid (D) spikelets. Bar = 1cm.

Anthers and ovary differs from haploid to diploid plants, and in haploid plants these structures were smaller and have an atrophied form (Figure 37).

Figure 37 – Phenotypic differences between haploid and diploid sexual elements. A: anthers from diploid (D) and haploid (H) plants. B: ovaries from diploid (D) and haploid (H) plants. Bar = 2mm.

Observing the structures on the figure 37 it is clear that a diploid chromosome set is essential to the normal development of functional sexual structures. Haploid plants, with only half of the genetic patrimony, are sterile, and pollen could not be observed within the anthers (Fig. 38).

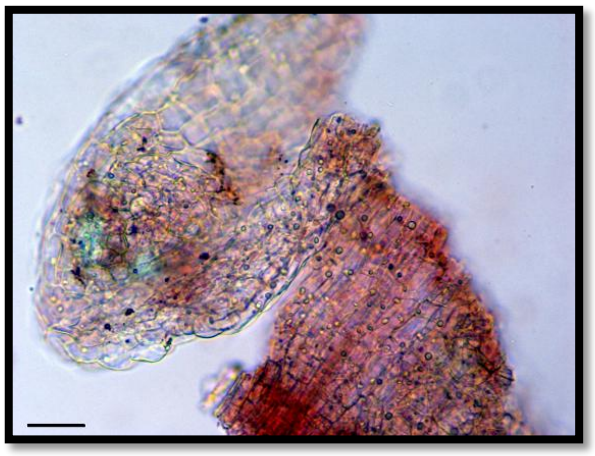

Figure 38 – Haploid anther tissue. Anther tissues without any pollen grains. Bar = 50µm.

Diploid sexual structures and pollen grains seem to be phenotypically normal and well-developed (Fig. 39).

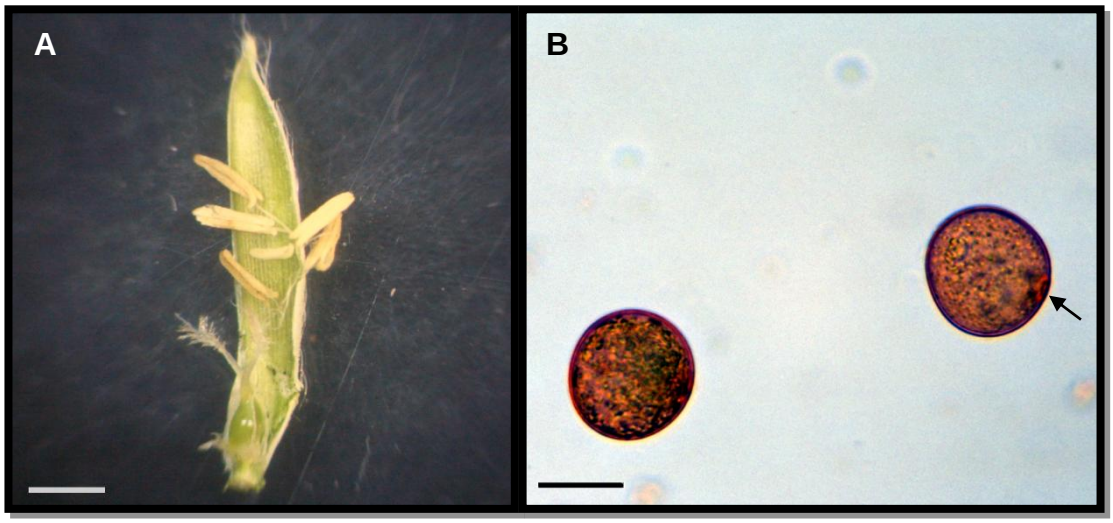

Figure 39 – Spikelet dissected from a diploid plant and pollen from the anthers. A: dissected spikelet of a diploid plant with well-developed sexual structures. Bar = 2mm. B: pollen grains “squashed” of diploid plant anthers, they seem to be normal reticulated grains with a single pore (indicated by the arrow). Bar = 50µm.

Most of diploid plants produced panicles approximately after 3 to 4 months in controlled conditions, and seeds are produced in some spikelets. These seeds appear to be completely normal and viable, presenting a normal embryo (Fig. 40).

Until now none of the haploid and triploid plants produce any seed, some of the haploid plants formed panicles, but not a single seed was produced.

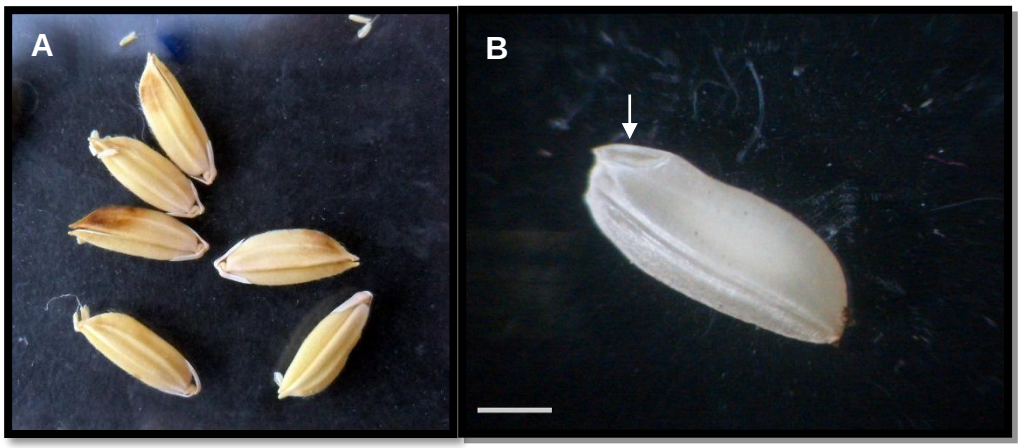

Figure 40 – A: Diploid caryopsis. B: Diploid seed collected from a diploid regenerated plant, showing the embryo (arrow). Bar = 2mm.

4. Discussion

Discussion

Androgenesis in cereals, mainly in rice, has been studied over the years, and efforts to make this technique a powerful tool in rice improvement are being made across the world (Gosal *et al.*, 1997).

Many researchers have tried to improve haploid production through androgenesis, mainly in crop species, applying different pre-treatments to the plants and evaluating the diversity of factors that control the process (Germanà, 2011; Islam and Tuteja, 2012). Rice was one of the most used species to obtain haploids through anther or pollen culture, and since the first report by Niizeki and Oono (1968), several studies were carried out to obtain the ideal protocol to achieve rice haploid production and homozygous lines. Genotype influence, microspore stage, effects of various treatments to rice donor plants, are some of the features tested in this studies (Chen *et al*., 2001; Trejo-Tapia *et al*., 2002; Herath *et al*., 2009; Khatun *et al*., 2012).

In this work, the androgenic potential of different breeding lines and cultivars growing in the Mondego valley were tested in order to evaluate possible improvement of rice culture in this area and in Portugal.

4.1. Androgenesis induction

Through this work, the induction of androgenesis, was always made when microspores were at middle or late-uninucleate stage. Androgeneis success rarely exceeds 1% for the majority of species, including rice, although the results of this work, had often shown induction rates higher than 2%. Similar results, have been reported by many researchers such as Cho and Zapata (1990) and Chen *et al*. (2001), who also obtained good induction rates when anthers were cultured containing pollen at the same maturation stage.

During anther culture, a few of the larger microspores, highly vacuolated, represented the portion that suffered divisions and initiate the androgenic route, whereas smaller microspores, sometimes with starch grains, usually do not underwent androgenesis and finished to die. Progressive decrease in total pollen viability during culture was also observed by Cho and Zapata (1990) and is a common feature of the androgenic process.

Over the years, studies on androgenesis have proved that sucrose concentration and hormone type and concentration on the induction media can highly influence the whole process (Trejo-Tapia *et al.*, 2002; Shahnewaz *et al.*, 2004; Islam *et al.*, 2004). Our results also showed that these two factors are important for the success of pollen embryogenesis.

The A3 medium proved to be the most effective for androgenesis induction, although statistical significant differences could only be found between A2 and A3 media. A3 was the only medium supplemented containing NAA, which seems to indicate that this auxin may have a positive role on the induction mechanism. This auxin was also used and proved to be a highly useful PGR in the assays carried out by Woo and Huang (1982) in rice. By the contrary, 2,4-D which was present in all media, does not seem to be so effective as NAA for pollen embryogenesis.

2,4-D has been used for androgenesis induction in a wide range of species and the best concentration to trigger the androgenic pathway seems to be different among the species. For example, Chen *et al*. (2001) reported that higher or lower concentrations beyond the optimal10-15µM, slightly decreased pollen callus formation. These results seem to indicate that some synergism between PGR, namely auxins and cytokinins, and eventually other factors (e.g. stress conditions) are responsible for the initiation of the androgenic pathway (Gupta *et al*., 1989).

The results indicated that sucrose is not a crucial factor for androgenesis in the rice genotypes that were tested. A previous report by (Chen, 1977) gave similar results although this author had claimed that a 6% sucrose concentration resulted in remarkable increases in the frequency of callus production, what did not occur in the present study. However sucrose level was not tested singularly, and the type and concentration of hormones in the media could be, and most likely was, determinant in these results.

An interesting feature of the results obtained in this work was the observation that during the three years of the experiment the breeding lines consistently gave better results than the cultivars. This may be caused by the great genetic diversity. However, more assays need to be carried out to more strongly support the role of the genotype.

Another interesting result is that different generations respond differently to androgenesis induction. In 2011, F2 generation lines had a higher induction rate than F3 lines, and the same happens in 2012, where F4 lines presented better results than F3. This may be also related to the genotype of the donor plants but, as stated before, needs further elucidation.

In 2012, F2 lines were not collected, being replaced by F4 lines. Studying the generation effects on the induction of androgenesis, it appears, by the results recorded in 2011 and 2012, that F3 is the generation that had the smaller induction response, being less susceptible to androgenesis induction that both F2 and F4 generations. Another prominent point, is that in 2012 the F3 descendants of F2 lines from 2011, gave in general, better induction results. This fact is not clearly understood and further studies on the genotype and its changes over generations are needed. Nevertheless, a possible cause for the response differences over the years could be related to the growth conditions (temperature, water supply….etc.) of the donor plants.

In 2012, another unexpected result occurred, this time among cultivars. Ariete, that on previous years produced pollen calli, could not be induced during this year experiments. The growth conditions of the anther donor plants may again be related with this result.

These results are identical to numerous studies on rice androgenesis, where it is proved that genotypic differences have an important role in conditioning the ability to microspore, even in the uninucleate stage, to rise embryoids and consequent haploids, as Guha-Mukherjee (1973) first reported.

The effect of anther tissues in androgenesis induction is also an important characteristic of the process. Pelletier and Ilami (1972), conducted a series of transplantation experiments and demonstrated that pollen from one cultivar of tobacco would successfully develop into an embryo even transferred into anthers of another cultivar. This work introduced the concept of “Wall factor”, according to which the somatic tissues of the anther play an important role in the induction of sporophytic divisions in pollen, with the diffusion of nutrients through the anther walls often considered to be one of the factors affecting androgenesis due the growth-inhibiting substances leaking out of the degenerating anther tissue. The main active tissue in the anther wall is the tapetum, and all nutrients to the developing pollen grains must pass through it

or be metabolized in it (Nitsch, 1977; Vasil, 1980; Bhojwani and Razdan, 1996; Germanà, 2011). Pollen grains are surrounded by the anther which is a barrier to direct contact with the culture medium, so in the interrelation of pollen and culture medium the anther wall should be seen as a bioactive filter (Heszky and Mesch, 1976).

A number of studies on the role of the anther wall in androgenesis have shown that it not only acts as a barrier to nutrient flow but that it also provides both beneficial and inhibitory substances (Heszky and Mesch, 1976). Some studies on this subject indicate that pollen grains do indeed experience a period of starvation within excised anthers, as the anther wall obviously act as a barrier to the flow of nutrients, such as sugar, amino acids and minerals, from the culture medium to the pollen. These conditions seem to stimulate the abnormal divisions of the microspores. Starvation appears to be the principal factor triggering tobacco pollen grains into androgenesis (Heberle-Bors, 1989).

4.2. The effects of cold-pretreatment

Cold-pretreatment at 4ºC for 10 days, on four lines and two cultivars (2012 assays), showed a positive effect on the androgenic induction. Most of the lines and varieties tested presented a higher induction rate on cold-treated samples in both A1 and A3 media. Although, lines 2523 and 3521 gave a better response in control than after the cold-treatment, the positive effect of the treatment is clearly visible on the response of cultivar Ronaldo. This variety do not respond on both media in control. However when suffering the cold shock calli production occurred, and with high rates of induction. Nonetheless, Ariete, one of the varieties also subjected to a cold-treatment period, did not show any response.

Temperature stresses, in particular cold treatments, have been used over the years by many researchers to promote androgenesis in rice. The results in this work are in line with those obtained by several authors that proved that the induction of androgenesis *in vitro* was strongly influenced by cold-pretreatment of the panicles, and sometimes increased callus producing 50% over that in control. This results could be explained by the possible delay of the senescense of anther somatic tissues, delaying the degeneration of microspores and thus

inducing pollen mother cell to produce two identical nuclei instead of one vegetative and one generative nucleus, allowing the androgenic route to take place (Zapata *et al*., 1982; Ying *et al*., 1982; Trejo-Tapia *et al*., 2002; Reddy *et al*., 1985).

4.3. Plantlet regeneration

Plantlet regeneration is one of the most important steps of haploid production, and through the years this process has been optimized, in order to take the maximum yield from the process (Sah and Niroula, 2007; Bagheri and Jelodar, 2008; Tariq *et al.*, 2008).

In this work, four different regeneration media, with changes in hormone composition, were used. Three of them had exactly the same hormones, NAA and kinetin, but at different concentrations. The other one, D3R, was the only medium in which BAP was tested. It has been claimed by many reports that NAA, is of extreme importance in the regeneration medium, since maximum regeneration rates are usually obtained through the addition of this auxin (Tsay *et al*., 1982; Woo and Huang, 1982;).

RR1 medium gave the best regeneration rate, opposite to RR3 medium. This shows that, although both NAA and kinetin are important in regeneration, their levels in the media have a high influence in the success of the process. A close correlation between the two PGRs was observed, and even a slightly change in their concentrations can positively or negatively impact the regeneration of plantlets.

These results support the idea of synergism between the PGRs, and that there is an optimum level for both PGRs, and that their effect could be supplemented by each other, creating an ideal balance that allow a maximum regeneration yield. In our work NAA concentrations lower that 1 mg/L resulted in a decrease of plantlet regeneration, and the addition of kinetin stimulated regeneration at concentrations up to 2 mg/L. Similar results were obtained by Tsukahara and Hirosawa (1992), even though they had the highest number of regenerated plantlets with the combination of 2 mg/L NAA and 1 mg/L kinetin.

The genotype is important not only for androgenesis induction but plays also an important role in controlling regeneration. Following the same trend

observed in callus induction, breeding lines gave higher regeneration rates than cultivars and, in 2012, only one cultivar, Eurosis, was able to regenerate plantlets. Although induction and regeneration responses of different genotypes to media is strongly governed by genetic and environmental factors, the composition of the media and a balance of auxin and cytokinin also influences the responses of the different genotypes (Karim and Zapata, 1990; Chaleff and Stolarz, 1981; Reddy *et al*., 1985).

Another significant factor that may control the success of the androgenic response, is the timing of callus induction from anthers and subculture on the regeneration media. This seems to influence the breeding efficiency, and callus in earlier stages not only possessed higher differentiation ability but also produced more green plants. In this work, a close correlation between the age and differentiation ability of callus could be found, being observed that young calli produce more green plants. Similar observations were reported by Tsay *et al*. (1982).

Calli initiated from microspores of rice are usually friable, shiny white, and nodular in nature (Sathish *et al*., 1995). This description is consistent with our observations and is an indication that callus are produced from microspores and not from anther wall somatic tissues. As the calli aged, it seems that part of the cell population turned non-embryogenic, losing their ability to differentiate into green plants as also observed by other authors (Tsay *et al*., 1982; Sathish *et al*., 1995).

Regeneration started about two weeks after callus transfer to the regeneration media and both green and albino plants were obtained. Some of the calli differentiated only into albino plantlets whereas others yielded exclusively green plants. In addition, a small number of calli, produced both albinos and normal plants in the same test tube. Similar results were reported by Woo and Huang (1982). The formation of green and albino plants in the same callus indicates that cells in the callus are quite heterogeneous in nature and possess different genetic backgrounds resulting in plantlets with different phenotypes after expression of totipotency. A callus mass either generated only one or two plants or in some cases large number of plants were produced. Moreover, a number of calli produced only roots.

Albino plants are a current problem in androgenesis in cereals. In our experiments, although a large number of albino plants were produced, especially in the experiments carried out in 2010, in 2012 experiments this problem was substantial reduced, and the number of greens plants were considerably higher than the albino plants.

Albino plant formation is known to be affected by several factors, although the exact mechanisms underlying this process have not been completely understood. However, the results in 2010 and 2012 could be explained by genetic differences between the lines and cultivars used, since the genotype is known to be a critical factor in the process. On the other hand, the reduction of albino regeneration and consequent higher yield of greens plants in 2012 may due to the cold-treatment applied before the culture, since some studies have shown the positive effect of this pre-treatment not only in callus induction but also in green plant regeneration (Sathish *et al*., 1995; Reddy *et al*., 1985; Woo and Huang, 1982).

RR3 medium showed the lowest regeneration ability, nonetheless, albino plant production was higher in this regeneration medium. This fact indicates the strong influence of hormones in the regeneration process, not only influence the plant regeneration itself, but also the type of plants regenerated. The hormone levels used in this medium (NAA – 1 mg/L and kinetin – 2 mg/L) must trigger some genetic feature changing the process, causing albinos regeneration. RR1 gave opposite results with the lowest number of albino plants regenerated and the highest rate of green ones, showing once again that could be a useful regeneration medium in the optimization of rice androgenesis. Some albino plantlets have been maintained in vitro and grow well since sucrose is present in the culture medium

When developing plantlets were transferred not to a hormone-free medium, but to a fresh regeneration medium of the same composition it was found that new plantlets arise, thus increasing the efficiency of the process. On these conditions, a single plantlet could gave origin to more than 20 new ones. Plantlets thus produced seem to emerge from shoots originated at the basis of the original plantlet, in a process resembling tillering in field conditions. Whether plants have origin in pre-existing meristems or are the result of newly formed meristems in an organogenic process remains unclear.

The regeneration results have shown that experimental lines presented a higher response than cultivars. For instance, in 2012, only Eurosis cultivar has the ability to regenerate plantlets. Among lines, it seems that donor plants from the F4 generation are the best for plant regeneration, with a higher number of formed plants, and lines 3521 and 3523 not only have a high rate of regeneration but also, all the plants regenerated from these two F4 lines showed to be diploid. This could be a very important condition, since F4 lines appears to spontaneous double their chromosome set during culture, although further studies must evaluate the plant origin.

4.4. Flow cytometry analysis

From the 114 plantlets that survived to acclimatization, it was found that haploid plants was the prevailing condition (77.19%). In other works the ratio haploid/diploid plants is different, and opposite to the results obtained in this work, Woo and Huang (1980), regenerated a higher number of diploid plants instead of haploid ones. The results obtained in this work by flow cytometry analysis also show that calli were obtained mostly from microspores and not from the somatic anther tissues. Despite the high number of haploid plants regenerated, diploid plants were also found (20.18%). It can be argued that these plantlets may be produced from anther somatic calli. However, as has been reported in other experiments with rice (Woo *et al.*, 1973; *Woo* and Huang, 1980) and with other species (Zheng, 2003), these may be also of pollen origin and may be the result of cell fusions at earlier or more advanced stages of androgenesis induction, as the regeneration of triploids indicates.

In rice, as in other cereals, the main mechanisms of spontaneous doubling of the haploid chromosome complement are endoreduplication (chromosome reduplication during interphase) or endomitosis (mitosis without formation of mitotic spindles) before the first microspore division (Chen and Chen 1980, Chen and Wu 1983). This characteristic could indicate that the diploid plants regenerated in this study were from microspore origin and that during the callus culture the chromosome number was doubled, which was also observed by Woo *et al*. (1973). Further analysis are needed in order to confirm these possible origins.

Flow cytometry analysis revealed that plants derived from the same callus presented the same ploidy status. This is complemented from the phenotype observation, thus these plants have identical phenotypic characters between them.

Callus flow cytometry analyses have shown that over two to three subcultures, ploidy condition suffered constant variations, and this could be associated to their regeneration ability loss. Rice microspore callus behavior during subculture is not well known yet, still it has been shown that this type of *in* vitro culture are generally characterized by instability of chromosome number and structure. During callus culture chromosome instability may occur, causing not only decrease in plant regeneration but also the elimination of haploid cells (Chen and Chen, 1980).

4.5. Phenotype analysis

As could be expected, haploid and diploid plants displayed different phenotypic characteristics such as size and flowers. The fertility of regenerate plants varied from plant to plant. Until now, all haploid plant that have completed full development were sterile, showing small panicles and abnormal sexual structures. Similar results were obtained by other researchers, and some of them only obtained seeds from haploid plant through a colchicine treatment (Woo *et al*., 1973; Woo and Huang, 1980).

Seeds from two diploid plants have been already recovered, and the formation of phenotypic normal panicles and spikelets in another ones, foresees the possibility of further recover of new seeds. Triploid plants do not show any type of panicle formation so far.

5. Future perspectives

Future perspectives

Haploid plants have been considered to be useful materials for genetic studies and plant breeding. Since only half of their genomes is involved, the function of recessive genes and the interaction of alleles can be studied easily. Nonetheless, the frequency of haploid occurrence is very low, and some treatments, such as cold-pretreatment have been used with some success over the years to enhance androgenesis. Although some treatments could increase haploid formation, genotype is a very important factor, and every treatment must be studied for each genotype individually.

Another important problem in androgenesis, the albinism, has been studied by several researchers in order to evaluate the causes and consequent possible solutions, and some new perspectives over this feature opens new doors to a possible control of albino formation. However, albinism still remains a problem, and although some treatments can reduce the number of albino plants, those are constant in every study in rice androgenesis made so far.

Culture media, both induction and regeneration, are an important factors that influence the whole androgenic process. Media elements, such hormones or sucrose are critical for the success, or not, of the culture. Induction results shows that auxin 2,4-D is essential to initiate callus formation and the association with NAA and kinetin appears to be important for induces androgenesis. A sucrose level of 3% showed to be the most effective in this study: However, further studies must be performed to evaluate the positive effects of sucrose 6% or higher concentrations both in terms of induction and regeneration as well on the rate of green to albino plant formation.

The time at which callus must be transferred from the induction to the regeneration media is very important, since a long exposure to induction conditions can prevent the regeneration ability. This suggests that in further works, a maximum possible number of calli should be placed in regeneration media a few days after their formation in order to maximize the number of regenerants.

A remarkable output of these assays was the regeneration of new plantlets from a single one when it is placed in a medium with hormones, usually in the same regeneration medium. In some cases, it was observed that

a single plant could originate more than 20 new plants, these new plants seems to be genetically and phenotypically identical, and ploidy analysis indicates the same chromosome set. This discovery needs further studies, but unlocks new possibilities to increase plant regeneration yield from androgenesis, one of the major problems of this technique.

New approaches in regeneration should be taken, and hormones could play a crucial role, not only in regeneration of plantlets from callus, but also in multiplication of single new plantlets, increasing the number of haploid plantlets and also spontaneous diploid plants. This could be a major breakthrough in androgenesis not only in rice but other important crop species.

Although rice breeding programs in Portugal remain mainly conventional, new approaches could be applied in a closed future, and Bico da Barca experimental field in Mondego valley is one of the most promising sites for rice breeding in association with *in vitro* techniques that could, and should create a new era in rice cultures in this country, in order to achieve the same success that in other European countries.

6. References

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