A Mediterranean japonica rice (*Oryza sativa*) cultivar improvement through anther culture.

X. Serrat [1,2], M. Cardona [1], J. Gil [1], A.M. Brito [1], L. Moysset [2], S. Nogués [2], E. Lalanne [1]*

(1) Germplasm platform, Oryzon Genomics, S.A., Cornellà de Llobregat, Spain.

(2) Departament de Biología Vegetal, Universitat de Barcelona, Barcelona, Spain.

* Corresponding author: xserragu8@alumnes.ub.edu
  Phone (+34) 934 033 717; Fax (+34) 934 112 842

Abstract

Certified seed producers systematically select and propagate registered varieties year after year in order to maintain their uniformity and the original registered cultivar traits. However, natural mutations, spontaneous breeding between varieties and alien grain contamination can introduce undesirable variability. NRVC 980385 is a temperate japonica rice cultivar (*Oryza sativa ssp. japonica*) first registered in Spain in 2002. In 2005 certification tests detected a plot differing from the original traits in terms of uniformity and height suggesting the presence of a certain heterozygosis. This material was therefore seen as an opportunity to obtain newly stabilized doubled haploid (DH) lines which could compete in the Spanish short grain seed market. In this study, an *in vitro* anther culture protocol is defined which also covers the field tests selection to obtain four new, improved and stabilized DH derived lines ready to be registered for commercial proposes. This took just four years from the initial anther collection until new lines were grown in large scale field trials. Consequently, this protocol reduces the time for obtaining field assessed DH lines thereby having considerable advantages over other techniques by both maintaining the original registered cultivars and/or generating new derived varieties.

Key words: In vitro, Anther culture, Rice, Dihaploid, Doubled haploid.

Introduction

In Spain, artificial breeding varieties obtained through genealogic selection of ancient Spanish varieties and artificial breeding (Herruzo 1986) started to displace traditional varieties and acclimatized foreign varieties about 1940. In the 60's new semi-dwarf varieties were obtained to respond to the new production techniques based on mechanization (Campos et al. 1966). In 2000, rice varieties obtained by breeding coupled to genealogic selection started to compete with others obtained through more a selection techniques such as molecular marker assisted-selection and doubled haploid (DH) selection. This technique help plant breeders to obtain new varieties through a more precise and faster characters selection, and consequently, lots of new genetically improved rice varieties had appeared, reducing cultivar lifetime in the Spanish market (Català et al. 2007; Català et al. 2009).

The anther culture technique was first developed in rice by Niizeki and Oono (1968). This technique allows one to obtain completely stabilized DH plants which bypass the inbreeding process (Brar and Khush 2006; Germana 2011). It is the fastest method for DH production as it only takes between 8 to 9 months (Agache et al. 1989). This technique manipulates the male sex cells in immature anthers, to induce haploid *callus* formation, which are subsequently converted to double haploid embryos (Niizeki and Oono 1968). Genetic recombination occurs during haploid sex cell production so that each microspore (immature pollen) which is produced is genetically unique. Consequently, each DH line obtained in this way will produce a new stabilized and unique line. This breeding tool has been used not only to establish parental pure lines saving the long inbreeding process, but also to speed up descendant's selection after an artificial cross, bypassing the classical pedigree selection process (Martinez et al. 1996; Courtois 1993; Moon et al. 2003)

Transgenic pest and herbicide resistant lines have been also developed in public institutes using Spanish rice varieties (Coca et al. 2004; Messeguer 2003; Marfa et al. 2002) with no commercial results to date, although foreign Clearfield herbicide resistant mutant lines have been recently introgressed into mid-grain rice and produced in Spain by *Cooperativa de Productores de Semillas de Arroz, S.C.L. (Copsemar, SCL)*. Its commercialization is expected in the near future, although the ecological concerns about the rapid resistance transmission to weedy rice reported (Shivrain et al. 2008; Kaloumenos et al. 2013; Goulart et al. 2012).

When a rice cultivar has been cultivated for many years, agronomic traits may begin to differ from the original traits as a result of natural mutations and spontaneous breeding between cultivars due to seed stock contamination in storage works, mechanical harvesters, seed dryers and transport, see Rice Seed Health (IRRI 1988). Certified Seed production programmes are currently working to counter this phenomenon through plot supervision and specific tests in order to guarantee the maintenance of its identity, genetic purity and physical, physiological and sanitary qualities (CIAT 1981).

The general suggested procedure for sowing panicle-rows or plant-rows (Briggs and Knowles 1967; Jennings et al. 1979) is commonly used to ensure the uniformity of breeder seed by way of the elimination of off-types that could result from residual heterozygosity. Breeder seed also called G-0 or Pre-Base 5 (PB5) plants are grown and usually 10 spikes collected from each breeder seed plant are sown in rows the following year, this is G-1 or PB4 seed. A single 10-row group showing the highest visual uniformity and fidelity to the original variety traits is selected by comparing data between rows and within rows. Only one row is harvested among the best 10-row groups, and its self-pollinated seedlings (called G-2 or PB3) are grown next year in a separate plot. At this point, several traits such as productivity and uniformity are supervised under pre-defined tests to select the Stage I foundation seed (also called PB2). This seed is then grown to obtain Stage II foundation seed (also called Base seed), and finally Stage II foundation seedlings are grown in order to obtain R1 and R2 certified seed to be sold commercially.

Different countries use slightly different procedures and tests to obtain similar results. This depends mainly on government regulation and the amount of certified seed needed to satisfy the market: see Silva et al. (1988), the Philippines; Rao (1988), India; Muñoz and Rosero (1988) and the book from *Centro Internacional de Agricultura Tropical* (CIAT 1981), Colombia; Masajo et al. (1988), Colombia and Africa; Botchey (1988) and Asea et al. (2010), Africa; *Boletín Oficial del Estado* (BOE 2010), Spain; INIA report, Venezuela (Torres et al. 2006) and; in general, Rice Seed Health from the International Rice Research Institute (IRRI 1988).

NRVC 980385 is a temperate japonica rice (*Oryza sativa*) cultivar derived from a traditional cultivar called Bahia which in turn was derived from the Balilla cultivar (Herruzo 1986). It was registered in 2002, and certified seeds have been produced and distributed by the rice farmers

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cooperative called *La Càmara Arrossera del Montisà*, SCCL (from now on La Càmara). Since then it has been grown in the Ebro River Delta at high and constant percentages when compared with other japonica varieties (Català et al. 2007). It is acclimatized to Ebro River Delta and Albufera de València environmental conditions. It has good germinability in direct seeding and even under cold conditions (Català et al. 2010).

NRVC 980385 was the shortest rice cultivar grown in the Ebro River Delta from 2002 until 2005, but new shorter japonica varieties have appeared in the rice seed market since (Català et al. 2007). In the Mediterranean climatic region, the weather is usually windy and rainy at the end of the growing season; therefore NRVC 980385 is more easily affected by lodging phenomena than are shorter competitor cultivars. Reduction of plant height has traditionally been the main target in order to improve lodging resistance (Keller et al. 1999) and semi-dwarf lines were introduced to prevent lodging and increase yield during the "green revolution" (Keller et al. 1999; Khush 2001; Sasaki et al. 2002).

The objectives of this study are 1) to produce NRVC 980385-derived DH lines from a seed batch showing heterozygosity from an undetermined origin and 2) to select in controlled environment (i.e. greenhouse) and field conditions the best lines showing enhanced productivity, uniformity and short plant height, while at the same time maintaining traditional NRVC 980385 rice qualities and characteristics as demanded by the local seed market. In order to reach theses aims we developed an *in vitro* anther culture protocol that allowed us to produce in a short time four new improved and stabilized DH derived lines ready to be registered for commercial proposes.

Materials and methods

Plant material and growth conditions

NRVC 980385 is a commercial temperate japonica rice cultivar grown in the Ebro River Delta region in the south of Catalonia (Spain). Plant material was obtained from a highly heterozygous NRVC 980385 seed batch which was first detected during certification test processes in 2005 by the La Càmara seed producer cooperative. One hundred seeds were sown in stages between January and March 2007 in greenhouse conditions at the Experimental Fields Service at the University of Barcelona (Fig. 1a). Plants were grown in 4 litre plastic containers filled with rice substrate: Floratorf peat moss (Floragard Vertriebs, Oldenburg) - vermiculite (2:1 v/v) substrate

supplemented with Osmocote (The Scotts Company LLC, USA) controlled-release fertilizer mix [Osmocote Exact (15 + 9 + 9 + 3 MgO+ micronutrients) for 6 months and Osmocote high K (11 + 5 + 15 + 1.2 MgO) for 9 months (1:1), 1gr/ litre of substrate]. One gram $CaCO_3$ per peat litre was added to adjust the substrate pH (around 6).

Tiller cold treatment, spike sampling and disinfection

Tillers were selected in the booting stage, which is characterized by a panicle formation growing inside the flag leaf sheath (boot leaf). Distance between the atria of the last two leaves was previously correlated to the correct spikelet developmental stage for the NRVC 980385 cultivar, between 4 and 8 cm (Fig. 1b). Harvest time was from 8:00 to 9:30 as recommended by Chen et al. (1991). A three-step disinfection process was carried out as follows. Harvested tillers were soaked in 70% ethanol for 1 minute and rinsed four times with sterilized water. Surface sterilized tillers were then cold treated: 7 to 12 days at 7 ℃ in polystyrene bags and in darkness to further enhance *callus* induction (Trejo-Tapia et al. 2002a; Trejo-Tapia et al. 2002b; Cai and Chen 1984; Lentini et al. 1997). Cold treated tillers were surface disinfected again with 70% ethanol before dissection. Panicles were then obtained under sterile conditions from the tillers by removing the leaf sheaths (Fig. 2c). The third disinfection step was carried out before anther plating as follows. The first 41 bare panicles were soaked for 3 minutes in 70% ethanol and rinsed five times in sterile distilled water. While the last 196 panicles were sterilized by soaking in 10% sodium hypoclorite solution for 3 minutes which was supplemented with Tween 20 (30 drops L^{-1}) and HCl 35% (50 drops L^{-1}), and followed by 1 minute in 70% ethanol and rinsed 6 times in distilled water as described by Lentini et al. (1997). These were then plated.

Anther Culture

Anther derived *callus* induction was obtained using media Chu N6 (Chu et al. 1975) modified as follows: N6 standard salts and vitamins fortified with 1 g L^{-1} casein hydrolisate, 250 mg L^{-1} L-proline, 2 mg L^{-1} 2,4 dichlorophenoxyacetic acid, 1 mg L^{-1} kinetin, 500 mg L^{-1} 2-(N-morpholino) ethane sulphonic acid (MES), 30 g L^{-1} sucrose and 3 g L^{-1} Gelrite. Sterilin 90mm petri dishes (Sterilin LTD, Cambridge) were filled with 25 ml media after autoclaving. Microspore stage determination was carried out in different stage panicles and spikelets to enhance anther culture efficiency (Fig. 1d). Anthers samples from the first plated panicles were fixed daily in acetic acid–ethanol (1:3) with 2% $FeCl_3$ for 24 h, and stained with 2% acetocarmine for microspore stage

determination following Mercy and Zapata protocol (1986), however, this procedure was stopped after one week of plating anthers as results matched the expected microspore developmental stage ratios. Yellow to slightly green spikelets (Fig. 1c) were found to be related to the optimum rate of late uninucleate microspore developmental stage, matching results obtained by Afza et al. (2000) and Shahjahan et al. (1992). Panicles were placed on sterile paper and spikelets were dissected under sterile conditions (Fig. 1c). As described by Lentini (Lentini et al. 1997), spikelet tips were first held with forceps and then the basal part was cut in the lower third part so that the anther filaments were cut at the same time. Anthers from selected spikelets that came from the same panicle were plated in each petri dish (Fig. 1e). Petri dishes were sealed using Parafilm (Pechiney Plastic Packaging Company, Chicago) and incubated at 24ºC for 6 to 8 weeks until the first anthers to respond produced the first *microcalli* (Fig 1f). Anthers were cultured in darkness as light is not necessary to induce *calli* and in fact, darkness helps *calli* growth (Lentini et al. 1997). Anther plating was stopped as soon as the first *microcalli* had been obtained. *Microcalli* were transferred to fresh *callus* induction media and grown until 2 mm in diameter before transferring to *callus* regeneration media petri dishes.

Green plantlet regeneration was carried out using Chu N6 (Chu et al. 1975) standard salts and vitamins fortified with 1 g L^{-1} casein hydrolysate, 250 mg L^{-1} L-proline, 1 mg L^{-1} naphthaleneacetic acid, 2 mg L^{-1} kinetin, 500 mg L^{-1} MES, 30 g L^{-1} sucrose and 3 g L^{-1} Gelrite. IWAKI 94 mm petri dishes (Asahi Techno Glass Corporation, Amagasaki) were filled with 25 ml media. Regeneration was done at 25 ºC and 50 to 70 μmols m^{-2} s^{-1} fluorescent light under a 16/8 hours day/night photoperiod until plantlet formation occurred (Fig. 1g). Frequently, ploidy duplication occurs spontaneously in rice during the *in vitro* regeneration process; therefore no antimitotic compound was used.

Fully tiny formed R1 plantlets (0.5-3 cm length) were first transferred to hormone free MS (Murashige and Skoog 1968) media tubes; the custom made 17 cm long *in vitro* glass tubes were filled with 12 ml hormone free MS media: standard salts and vitamins fortified with 500 mg L^{-1} MES, 30 g L^{-1} sucrose and 2 g L^{-1} Gelrite for 2-3 weeks. Next, these R1 plantlets (10-15 cm) were then propagated using a propagation media (Fig. 1h) which is the same hormone free MS media except fortified with 0.5 mg L^{-1} 6-benzylaminopurine. Culture was carried out at 25 ºC and 50 to 70 μmol m^{-2} s^{-1} fluorescent light under a 16/8 hours day/night photoperiod and was extended

so as to have enough R1 plants to carry on the ploidy determination and additional assays (Figs. 1 and 2).

All media components were supplied by Duchefa (Duchefa Biochemie BV, The Netherlands). Medias were prepared using distilled water and the pH was adjusted to 5.7 by adding KOH (Sigma-Aldrich Co) solutions. All components including hormones were added before standard autoclave sterilization (120 ºC for 20 minutes).

Ploidy determination

About 1cm^2 of newly formed leaf tips from each line were placed on 55 mm diameter plastic petri dishes; the tissue was then sliced and chopped into small pieces by addition of 1.6 ml of lysis buffer and use of a razor blade and incubated for 14 minutes. Lysis buffer composition was 15 mM Tris-HCl pH: 7.5, 2 mM disodium EDTA, 0.5 mM spermine tetrahydrochloride, 80 mM KCl, 20 mM NaCl, 0.2% Triton X-100 and pH 7.5 adjusted with 1M NaOH. The homogenate was filtered through a 33 µm nylon mesh. Then, 15 µl propidium iodide was added to 600 µl of filtered solution and after incubation for 30-45 minutes at room temperature, the fluorescence intensity of nuclei was measured using a Coulter EPIC XL flow cytometer (Beckman Coulter, Miami, FL) at the Cytometry Unit (Scientific and Technological Centers, University of Barcelona). Summit Software v4.3 (Cytomation, Fort Collins, Colorado) was employed for data analysis. Two diploid NRVC 980385 samples were used as the reference ploidy controls where the controls produced the same single peak. Accessions were classified as DH if they produced a clearly defined single peak corresponding to the peak of the diploid controls. Accessions producing a half fluorescence intensity peak were classified as haploids (Figure S1). The main haploid R1clones were discarded such that only one R1 haploid individual per line was kept.

Propagation, *ex-vitro* Acclimatization and Seed Production

R1 DH lines were *in vitro* propagated in tubes for two months using propagation media (Figs. 1h and 2) until a minimum of 100 clones from each line were obtained. Propagated R1 clones were *ex-vivo* acclimatized as follows: media was carefully removed from the roots using tap water and plants were transplanted into 96-well multipots and each pot was filled with 35cm^3 of rice substrate (Fig. 1i). Recently transplanted R1 plants were placed in an acclimatization tunnel inside the greenhouse. Relative humidity was reduced over 100% to 70% for two weeks. After the

acclimatization process plants were transplanted to 4L plastic pots (3 plants per pot) filled with rice substrate and grown in greenhouse conditions as described in Plant materials and growth conditions, however, the temperature was maintained at 19°C. R1 plants were fertilized during the panicle formation period using high ammonium (50% Total N) soluble fertilizer (NPK 19+6+6) supplemented with 4% w/w micronutrients and 4% w/w iron chelate diluted in osmotized water with a final electroconductivity adjusted to 1200 micro Siemens. DH plants were harvested individually in May 2008 and R2 seeds from the most vigorous and shortest cycle lines were used for Experiment 1 in the summer (see below). A haploid individual from each haploid line was also acclimatized to check flow cytometric ploidy assay results. R1 plants were ratooned and fertilized again during the panicle formation period to obtain more R2 seed for further field trials (Table 1, Figs. 1, 2).

Experimental design of field trials

Field trials were carried out in La Càmara fields. All plots were at least 15 meters apart from one another in order to avoid cross pollination. Distances between field trial and other rice fields were always more than 20 meters. Standard fertilization and field management principles were applied. At least eight plants were randomly selected from each line and experiment in order to record data measurements. General traits such as the number of spikes per plant, different height measurements, grain/spike phenological stage, uniformity, pests/diseases and plant shape were recorded weekly. Other specific traits such as fungal lesions on leaves, flag leaf width, length and angle, fungal infection in spikes, days to heading, spike position and exertion, and late flowering ratio were also recorded weekly. Plants were harvested at maturity to obtain the yield results. Three different experiments were performed as mentioned below.

Experiment 1 was designed to test basic agro-morphological traits mentioned above and to select the most interesting lines using R2 seeds obtained from the initial R1 regenerated population. In the summer of 2008, the first 10 lines (DH1 to DH10) were tested. R2 seeds were sown in greenhouse conditions just three weeks after harvesting the original propagated and acclimatized R1 DH lines (DH1 to DH10). R2 plants were planted in the field late in the season, on June 2nd 2008. In summer of 2009, ten more lines (DH11 to DH20) were tested, plots were more homogeneous in number of plants and planting was done throughout the whole growing season (Table 1). In both years, three to four leaf stage plantlets were transported to La Càmara

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Experiment 1 field, being hand-planted with a spacing of 25×20 cm in small plots (Figure S2). NRVC 980385 plots were also planted in the same way and at the same time in order to compare results. This field was designed as follows: 18 plots (2 meters x 5 meters) were reserved for assessed lines and NRVC 980385 controls. The bottom margin of the Experiment 1 field corresponds to the 5 meters width access road; the distance from plots to the road was only 15 meters. A minimum 15 meters distance between lines and 20 meters to adjacent field was designed to avoid gene flow-mediated hybridization.

Experiment 2 field trials were carried out to test R3 seeds form Experiment 1 selected lines on a medium scale (Table 1). Parcels were planted by hand with a spacing of 25×20 cm using almost all the available R3 seed; with parcels ranging from 1242 m^2 to 4980 m^2 in size (Table 2, Fig. 1j). The NRVC 980385 controls were also hand-planted in parcels adjacent to each DH line using the same format and field conditions as experimental plots so as to be able to compare results. Experiment 2 was performed twice as follows (Table 1):

DH3, DH8 and DH9 seeds obtained from experiment 1 (R3) and certified NRVC 980385 seeds were sown in 2009 in greenhouse conditions and hand planted in three rice parcels to assess traits as described above. DH3, DH8 and DH9 lines were grown in 4480 m^2, 2333 m^2 and 4060 m^2 parcels respectively. Field size was mainly determined by seed availability.

In 2010, Experiment 2 was repeated using R4 seed from DH3, DH8 and DH9 lines, and likewise, R3 seeds from DH12, DH16 and DH18 obtained in Experiment 1 were also assessed. Six additional NRVC 980385 fields were used as controls, one control per line, each one growing next to each study line. In this experiment, a similar number of plants and plot dimensions were used thanks to seed availability. DH3, DH8 and DH9 were grown in 1830.4 m^2, 1830.4 m^2 and 1919.4 m^2 parcels respectively, and DH12, DH16 and DH18 were grown in 1325.5 m^2, 1242 m^2 and 1642.5 m^2 parcels respectively.

Experiment 3 was a large scale field trial (Table1, Fig. 1k). R4 seeds from DH3, DH8 and DH9 lines were machine-sown in different areas, soils and fertilizer conditions in order to test the yield performance of each line under real conditions using standard seed production practices. Farmers' observations were also taken into account although traits recorded in Experiment 1 and 2 were also recorded weekly in Experiment 3.

Two different fields were used per line. The fields were rented out by La Càmara to six different cooperative associate farmers. The sum of Ha per line in associate farmers' fields ranged from 4.5 to 4.8 Ha. Yield was tested and compared to the average NRVC 980385 yield obtained that year from seed production fields grown by La Càmara farmer associates.

DH3 was grown in 2.85 Ha and 1.93 Ha fields called Field No.1and Field No.2 respectively. Field No.2 was over fertilized. DH8 was grown in 3.57 Ha and 0.88 Ha fields called Field No.3 and Field No.4 respectively. Field No.3 was placed beside a NRVC 980385 registered seed production field and in this case control data was taken. Field No.4 was highly clayey and fertilized using natural guano. DH9 grown in 2.05 Ha and 2.41 Ha fields were called Field No.5 and Field No.6 respectively.

Results

Anther culture and DH lines

The DH lines obtaining from anther culture took a total of 233 days (Figs. 2 and S3). In our experimental conditions booting stage tillers were harvested late in the season, from June to September 2007 and in total 42,660 anthers originating from 237 spikes were sown over 53 days.

Sixty three out of 237 petri dishes were discarded due to contamination. Ethanol panicle disinfection resulted in a 40% plate contamination rate, while Clorox disinfection reduced contamination to 22.25%, but no *calli* were obtained from this second sterilization process.

Anther plating and *callus* induction was stopped on the 53rd day as soon as the first 4 anthers to respond produced the first globular and clear *microcallus* masses which were growing from different parts of the anthers (Fig. 1f). *Microcalli* grew during the regeneration process and produced green spots and small roots which were followed by shoot development (Fig. 1g). The first plantlets had fully formed 30 days after the regeneration process had begun (Fig. 2). The regeneration process yielded 42 green plantlets, 6 albinos and 1 green and white chimera. Regenerated green plantlets represented 85.71% of the total plantlet regeneration. Flow cytometry assaying identified 29 DH and 13 haploid lines (Fig. S1), thus DH lines represented 69% of total green plantlet lines. One chimeric white and green plantlet was also analysed and was found to be a triploid plant.

All DH green plantlet lines were efficiently propagated over a two month period giving a minimum of 100 plants per line. Plantlets were acclimatized and grown until maturity in greenhouse conditions. Acclimatized haploid individuals were dwarf and sterile as expected (Niizeki and Oono 1968), confirming flow cytometry results. DH line seedlings were harvested individually in May 2008 (Fig. 1) and plants were ratooned in order to obtain more seed for further experiments.

Experiment 1

The tiller per plant ratios were strongly increased in DH3, DH8 and DH9 lines (43%, 70% and 58% increases, respectively). DH3 was the shortest line, 15 cm shorter in comparison to NRVC 98038. This line did not look like NRVC 980385 as flag leaves were 41% longer. Line DH8 was about 10 cm shorter and flag leaves were only 16% longer. Furthermore, DH9 characteristics were most similar to those of NRVC 980385 but plants were only 8 cm shorter. Height results are shown in Figure 3a. Yield results were not taken into account since plots were too small and were of different sizes.

In 2009, the same Experiment 1 was carried out throughout the entire season, and 10 more lines, from DH11 to DH20 were tested. The number of plants per plot was more balanced. Height results were worse in comparison to results from the previous year (Fig. 3b). Finally, three lines were selected (DH12, DH16 and DH18) in accordance with yield, grain quality, plant height and other agronomic data (not shown).

Experiment 2

The first Experiment 2 performed in 2009 confirmed height results of DH3, DH8 and DH9 lines (Fig. 4a). In this Experiment 2, tiller number increase was not significant in comparison to NRVC 980385, but lines were shorter than NRVC 980385 with DH3 being the shortest line (Figs. 4a and 4b). Yield results were very similar between lines and NRVC 980385 controls (Fig. 5a).

In order to confirm these results, Experiment 2 was repeated in 2010 using seeds harvested from the DH3, DH8 and DH9 lines from Experiment 2 performed the previous year. In addition, seedlings from three more lines (DH12, DH16 and DH20) which were also selected from the second Experiment 1 (2009) were tested. Height results were confirmed again for DH3, DH8 and DH9 (Fig. 4b), while height reduction was poor in second set lines. DH12, DH16 and DH20 lines

were taller than controls, DH3, DH8 and DH9 assessed in the same experiment and in the same year (Figs. 4b and 4c), although height was almost acceptable for DH12 and DH16 (Fig. 4c). Yield results showed that DH12 had nearly 30% more productivity, while yields in all other lines were similar to NRVC 980385 controls (Fig. 5).

Experiment 3

DH3 was once again the shortest line and also the distance between the panicle collar and the first leaf was clearly shorter than in that of NRVC 980385. In Field No. 2, the farmer over fertilized the field. Therefore, plants were taller and greener than in Field No.1 but yield was reduced and a higher level of fungal attack and lodging was detected. DH8 was grown in Field No.3 surrounded by an NRVC 980385 registered seed production field managed by the same farmer, from which control data was taken. Here, an average height reduction of 13 cm was scored. DH9 was clearly shorter than NRVC 980385 but plant shape was similar. Yield results are shown in Table 2 and Fig. 5.

Discussion

An anther culture method based on the Lentini et al. protocol (1997) has been modified (see above) in our lab in order to efficiently obtain DH lines from NRVC 980385 cultivar plants grown in greenhouse conditions using spikes from plants sown in season which resulted in a high rate of *callus* production. However, in this study plants were sown from January to March, three months in advance of the normal growing season. Although the greenhouse was heated and NRVC 980385 is photoperiod insensitive, total irradiance was lower than in season and furthermore, 50 of the 100 donor plants had to be ratooned in order to obtain a second set of panicles. Ratooning plants and collecting spikes out of season could affect *callus* induction (Chen et al. 1991; Lentini et al. 1997; Raina and Zapata 1997; Guzman and Arias 2000; Heberlebors 1985), however Guzman and Arias (2000) reported higher green plantlet regeneration using ratooned Taipei-309 indica rice cultivar.

Although harvest time was from 8:00 to 9:30 am following Chen's recommendations (1991) and the collected spikes were cold treated to enhance *callus* induction (Trejo-Tapia et al. 2002a; Trejo-Tapia et al. 2002b; Cai and Chen 1984), success in obtaining DH lines through anther culture is highly related to the amount of cultured microspores in the middle to late uninucleate stage present in plated anthers (Chen 1977; Gupta and Borthakur 1987; Bishnoi et al. 2000). This microspore

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developmental stage has been correlated to the distance between the atria of the last two leaves, but may vary depending on plant genotype and the environmental conditions in which it is placed (Lentini et al. 1997). Several authors have studied this correlation to improve anther culture and green plantlet regeneration rates, each one using different cultivars; Chen and Chen (1979) recommended collecting tillers with a distance of 5 cm between the atria of the last two leaves in Tainan 5 indica cultivar; Abbasi et al. (2011) reported 4 to 8 cm while Herath and Bandara (2011) reported 3 to 7 cm, both using indica-japonica hybrids. Our investigations in NRVC 980385 cultivar prior to this present work concluded that a 4 to 8 cm distance between the atria of the last two leaves and the flag leaf had the highest ratio of yellow to slightly green spikelets which also contained the highest ratio of middle to late uninucleate stage microspores according to Afta et al. (Afza et al. 2000). In the present study, the developmental stage in microspores was determined only in the first panicles since they matched our previous results. Efforts were therefore directed towards sowing as many anthers as possible in order to ensure enough DH lines were yielded. Nevertheless, the relationship between atria distances and microspore developmental stages could have changed during the plating process as donor plants were sown out of season and some of them had been ratooned.

Contamination is quite common in anther culture as spikelets cannot be completely sterilised without affecting anther viability (Lai et al. 1980). In our study, the high average contamination rate (26.58%) in the anther derived *callus* induction process could be due to endogenous contamination and soft ethanol disinfection. This contamination rate also affected *callus* induction efficiency. The ethanol sterilisation process applied to the first 41 spikes resulted in a 40% plate contamination, thereby a Lentini's Clorox sterilization process was followed (Lentini et al. 1997) and contamination incidence was reduced (22.5%), however, no *calli* were obtained.

Although some authors succeeded in obtaining rice DH plantlets directly sowing anthers in one-step culture media without transferring to regeneration media so as to save time and resources (Karim and Zapata 1990; Marassi et al. 1993), two-step culture media protocols are widely used and described by authors (Lentini et al. 1997). As optimal media composition is highly affected by the genetic backup of the plants (Talebi et al. 2007) we adapted the protocol of Spanish Hispagran

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rice variety, genetically similar to NRVC 980385 (Wankhade et al. 2010), assessing N6 and MS salts and vitamins with different hormonal doses in order to induce anther derived *calli* formation and subsequent regeneration. N6 based *medium* yielded the best results in calli formation both in Hispagran and NRVC 980385, which agrees with results from other authors where the application of N6 medium is quite suitable for japonica rice, but usually not for indica rice cultivars (Reddy et al. 1985).

However, the anther-derived *callus* induction media for NRVC 980385 required few modifications on the previously established Hispagran protocol. The concentration of 2,4-D was reduced from 2.5 mg L^{-1} to 2 mg L^{-1}, while kinetin (1 mg L^{-1}) was maintain. This 2,4-D doses is in the range of that used by other authors (Shimada et al. 1999; Islam et al. 2004). Also, Herath et al. (2009) coincided assessing 2 mg L^{-1} 2,4-D and 1 mg L^{-1} kinetin in Hu lo tao japonica rice variety.

The use of 4% maltose as a carbon source was tested and rapidly discarded after different assessments with no *callus* formation although better results were obtained in indica rice by (Bagheri et al. 2009) using Iranian rice cultivars, (Niroula and Bimb 2009) using Nepalese varieties and (Javed et al. 2007) Indian rice cultivars. Proline was found to increase *callus* induction as reported by Cho and Zapata (1988), being 250 mg L^{-1} the optimal dose for both Hispagran and NRVC 980385 cultivars.

Albinism is a common problem in anther culture and generation of doubled haploids. Loss of chlorophyll pigments and incomplete differentiation of chloroplast membranes is partially influenced by environmental conditions, media composition and culture conditions, but genetic factors are the major determinants (Kumari et al. 2009; Yamagishi 2002). In our study, the proportion of albino plants among anther derived regenerated rice plants was only 14% which is quite low in comparison to 36% in the Nipponbare rice model cultivar (Yamagishi et al. 1998). Although, Wang reported that frequencies of albino shoot forming microspore *calli* range from 5 to 90% in different temperate japonica cultivars (Wang et al. 1981).

When *in vitro* anther cultures are performed, diploid plants are mainly homozygous DHs, but heterozygous diploids produced by the somatic tissue can occur, although in rice this is very unlikely (Chen et al. 1982). Some authors discard heterozygous diploids using isozyme analyses, RAPD markers, SCARs or SSRs to assess homozygosity (Germana 2011). In addition, conventional cytological techniques can be employed to determine the ploidy level of regenerated plants, but ploidy level can be more easily assessed by way of flow cytometry analysis (Bohanec 2003).

Our results indicated that plants were mainly DH (69%), although one polyploid and some haploid plants were also detected. The DH percentage obtained among green regenerated plantlets is similar (56.4%) to that obtained by Mercy and Zapata (1986). Consequently, flow cytometry helped us to discard non-DH plantlets thereby saving time and resources in *in vitro* propagation works.

In our case, field experiments resulted in high visual homogeneity in all offspring and no apparent character segregation was observed in any line.

A first selection was carried out when acclimatized plantlets were grown in greenhouse conditions until seed set. Only the first 20 DH lines producing seeds were tested in Experiment 1. All DH lines were stable and homogenous. Consequently, twenty healthy, stable and homogeneous DH lines having the shortest cycle were tested in Experiment 1 small field trials. Due to seed availability, the number of individuals per plot was from 50 to 120 plants in Experiment 1 performed in 2008. The number of tillers per plant appeared to be strongly increased in DH3, DH8 and DH9 lines (43%, 70% and 58% increases respectively) in Experiment 1, however, no significant increases in this trait were obtained from subsequent experiments. It was suspected that this apparent increased tiller formation was due to the fact that NRVC 980385 controls had more plants per plot and it decreased the border effect in comparison to DH line plots.

Selection criteria was initially mainly focused on reduced height, enhanced tillering and enhanced yield, although all other weekly measures data records such as different height measurements, grain/spike phenological stage, uniformity, pests/diseases, plant shape, fungal lesions on leaves, flag leaf width, length and angle, fungal infection in spikes, days to heading, spike position and exertion and late flowering ratio were taken into account. Six lines were selected from Experiment

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1, three from the 2008 Experiment 1 field trial, which were considered to be the most interesting,

and three more lines from the 2009 Experiment 1 which demonstrated insufficient height reduction but which had interesting yield results. These six lines were assessed on medium scale field trials (Experiment 2) and from these, the three lines obtained in 2008 experiment were assessed in real conditions large scale trials (Experiment 3) as they had obvious height reduction, high homogeneity and acceptable productivity. Nevertheless, the DH12 line had an exceptional and unexpected yield result.

Rice production and yield assays have to be repeated for at least three consecutive years and using replicates and control replicates, as field trial results are usually variable (Fig. 5). In our study, field dimensions were different in each experiment due to seed availability and they were placed in different locations due to field availability. Nevertheless, this three seed lines have been successfully submitted to Spanish registration process and other lines are still today under study.

Selected lines

The DH3 line differed from NRVC 980385 in a variety of traits: higher uniformity, shorter plants, shorter flag-leaf to spike distance (half the distance), wider and bigger spikes, however, with similar rice production. DH8 differed from NRVC 980385 in terms of early flowering and maturation (up to two weeks) and shorter height, although it had a similar yield. DH9 is highly uniform, shorter than NRVC 980385 but taller than the DH3 and DH8 lines. DH12 turned out to be the most interesting line as it was the most productive.

Conclusions

In conclusion, an *in vitro* protocol based on the anther culture technique has been designed and optimized to obtain DH lines for the Spanish Mediterranean temperate japonica variety NRVC 980385. This protocol allows one to move rapidly from the anther collection stage to large scale field trials, thereby achieving the production of field assessed DH lines in a much reduced time period. This can now be adapted to other temperate japonica rice cultivars in order to obtain improved lines, and therefore avoid long breeding and inbreeding processes. It can also be adapted to obtain selected DH lines from F1 or F2 hybrids.

Acknowledgements

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Tables

Table 1. Field size of different experiments and DH lines assayed during 2008, 2009 and 2010 (see details in Material and Methods).

Experiment	Field size	DH lines assayed	Years
1	Small	DH1 to DH10 DH11 to DH20	2008 2009
2	Medium	DH3, DH8, DH9 DH12, DH16, DH18	2009 and 2010 2010
3	Large	DH3, DH8, DH9	2010

Table 2. Yield data from experiments 2 and 3. *Average NRVC 980385 seed production yield in 2010. NRVC 980385 cultivar was used as control.

Line	Experiment 2 2009		Experiment 2 2010		Experiment 3 2010	
	Surface (m^2)	Yield (Kg/Ha)	Surface (m^2)	Yield (Kg/Ha)	Surface (m^2)	Yield (Kg/Ha)
DH3	4982.7	6966.16	1835.4	7273.62	47807.4	7299.77
DH8	2333.0	6763.82	1830.4	7659.53	44517.9	8106.16
DH9	4060.7	7368.12	1919.4	6892.78	44649.5	7091.98
DH12	n/a	n/a	1325.5	9038.10	n/a	n/a
DH16	n/a	n/a	1242.0	7181.96	n/a	n/a
DH18	n/a	n/a	1642.5	6295.28	n/a	n/a
CONTROL	10964.5	7753.20	5745.5	7019.41	n/a	6863.00*

Figure captions

Fig. 1. Obtaining *in vitro* rice DH lines and subsequent field trials. a) Donor plant material, b) shoot selection, c) spikelet selection, d) microspore stage determination, e) anther plating, f) *callus* grown from anther culture, g) plantlet regeneration from anther derived *calli*, h) plant propagation, i) acclimatization to greenhouse conditions, j) experiment 2 (year 2009) on DH8 line, k) experiment 3 (year 2010) on DH8 line, l) experiment 4 (year 2010) on DH8 line.

Fig. 2. Schematic representation of NRVC 980385 rice cultivar anther culture schedule to obtain DH lines and its propagation from June 2007 until February 2008. Total days (d) per step are indicated. Pd = Plantlet development Pa = Ploidy analysis took place in 5 days.

Fig. 3. Height average for Experiment 1 tested lines and NRVC 980385 controls in both 2008 (**a**) and 2009 (**b**) trials. Each bar is the average of at least 8 replicates ± standard error.

Fig. 4. Height average for Experiment 2 tested lines and NRVC 980385 controls in (**a**) 2008 trial and (**b** and **c**) 2009 trials. Each bar is the average of at least 8 replicates ± standard error.

Fig. 5. Yield data from primary selected lines. Experiment 2 data from 2009 assay (**a**) and 2010 assay (**b**) is compared to NRVC 980385 control grown by the lines and cultured the same way. Experiment 3 data (**c**) is compared to NRVC 980385 average production in seed production fields in the same year.

Supplementary data

Figure S1. Coulter EPIC XL flow cytometer results using Summit Software v4.3. **a** dihaploid sample, **b** haploid sample.

Figure S2. Schematic of the design of the 2008 (a) and 2009 (b) Experiment 1 field trials. Eighteen 10 m^2 plots (2 meters x 5 meters) were reserved for assessed lines and NRVC 980385 controls. Their position is indicated. The bottom margin of the field corresponds to the 5 meters width access road, the distance from plots to the road was only 15 meters. A minimum 15 meters distance between lines and 20 meters to adjacent fields was designed to avoid gene flow-mediated hybridization.

Figure S3. Anther culture and lines tested in the study.