

Short Communication

# An improved anther culture procedure for obtaining new commercial Mediterranean temperate *japonica* rice (*Oryza sativa*) genotypes

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**Abstract** Rice is one of the greatest calorie supply for the world population, especially since its production is almost entirely destined to direct human consumption and its demand will increase along with the world population. There are efforts worldwide to increase rice yields by obtaining new improved and stabilized rice lines. The rice anther culture, a fast and cheap technique, allows to obtain double haploid lines in less than one year. We report its application with an improved protocol in four Mediterranean *japonica* rice genotypes at F<sub>2</sub> generation. We performed a screening test for cold-pretreatment at 5.0±0.1°C and concluded that the optimum duration was 9 days as it produced the higher rate of anther-derived *callus* induction. This revised protocol was successfully applied to the four genotypes, obtaining good results in all the procedure's steps. At the end, more than 100 of double haploid green plants were generated. Moreover, 9 lines obtained from the anther culture procedure showed good qualities for the Spanish market at the growing, farming and grain production level during the field assays. Therefore, we report an improved anther culture procedure for obtaining double haploid lines from temperate *japonica* rice genotypes showing high commercialization expectance.

**Key words:** anther culture, cold-pretreatment, field assays, Mediterranean rice, *Oryza sativa*.

Rice is a highly important cereal cultivar in the world, with a total of 490.9 million tonnes (milled equivalent) produced in 2015/16 of which more than 80% was destined to direct human consumption (FAO Trade and Market Division 2017). In addition, it has been proposed that rice will be one of the main calorie supplies in the forthcoming years (FAO Rice Market Monitor 2016). Thus, there are efforts worldwide to accelerate the development of new rice varieties either to attain higher yielding rates and/or to obtain higher quality grains (Guimaraes 2009; Khush 2005; Moon et al. 2003; Peng et al. 2008; Zeng et al. 2017). Despite the efforts made, rice breeders' seed suffer recurrent deteriorations due to successive annual cultivation (IRRI 1988; Serrat et al. 2014). Programs for ensuring rice breeders' seeds stability are laborious and time-consuming (Briggs and Knowles 1967; Jennings et al. 1979; Serrat et al. 2014). In addition, selecting and stabilizing new rice lines from an F<sub>1</sub> cross is a long process that usually takes about 8 years minimum (Martínez et al. 1996; Serrat et al. 2014).

The anther culture technique, first developed in rice by Niizeki and Oono (1968), allows to obtain stabilized

double haploid (DH) plants bypassing the inbreeding process. Moreover, it is the fastest method to obtain DH rice plants as can be performed in less than one year (Agache et al. 1989; Miah et al. 1985). Roughly, this technique is a two-step process from the initial development of *calli* to the subsequent regeneration of green plants from embryogenic *calli* (Mishra and Rao 2016). This technique has been used to obtain pure parental lines and to speed up descendant's selection after an artificial cross (Courtois 1993; Mishra and Rao 2016). Over the years, it has been shown that it is much easier to apply this technique on tropical *japonica* varieties, since they are more responsive at the *callus* formation and plant regeneration stages than Mediterranean *japonica* or *indica* varieties (Chen et al. 1986; Herath et al. 2007; Hu 1985; Miah et al. 1985; Mishra and Rao 2016; Serrat et al. 2014; Yan et al. 1996). Despite of that, we have previously reported an anther culture technique adaptation for a Mediterranean temperate *japonica* rice (*Oryza sativa*) cultivar (NRVC980385) to produce a new commercial cultivar (NRVC20110077; Serrat et al. (2014)), which however showed a very poor anther-derived *callus*

induction.

Therefore, the main aim of this study is to test for the first time and improved anther culture procedure on  $F_2$  rice genotypes coming from self-pollination of four crosses between different temperate Mediterranean *japonica* rice varieties. In addition, a secondary aim was to test the effect of a colder cold-pretreatment performed at different days of exposure for increasing the anther induction rate. This will allow to establish a standard and fast technique for obtaining commercial DH plants, with a high anther induction efficiency, from any temperate Mediterranean *japonica* rice line in development.

For testing this improved protocol, four different  $F_2$  rice genotypes that resulted from self-pollination of an  $F_1$  generation generated by crosses between Mediterranean temperate *japonica* rice cultivars were used (Table 1; germplasm rice genotypes were coded according to La Càmera cooperative seed producer simplified coding system). We employed the  $F_2$  generation as characters segregation is maximum and plants will therefore provide high variability when obtaining the double haploid green plants (Guimaraes 2009). Plants were grown in greenhouse conditions at the Experimental Fields Service at the University of Barcelona (Barcelona, Spain) on four litre plastic containers filled with rice substrate as described in Serrat et al. (2014).

The anther culture procedure was performed similar to Serrat et al. (2014). The cold-pretreatment was modified in order to enhance the anther-derived *callus* induction stage according to (Chen et al. 1986; Trejo-Tapia et al. 2002a, 2002b). We performed a screening test at  $5.0 \pm 0.1^\circ\text{C}$  during 8 to 12 days to select the best cold-pretreatment duration for using it for the anther protocol. Haploid *calli* spontaneously double their ploidy during the plantlet regeneration step, and thus develop into double haploid (DH) plants but could also develop into haploid, triploid or polyploid plants (Alemanno and Guiderdoni 1994). Further, the ploidy level was analysed with the aim of reducing greenhouse space and costs since haploid plants are sterile. The ploidy determination was performed by flow cytometry following the protocol described in Serrat et al. (2014). Dihaploid plants were cultured in greenhouse until seed-set, and seeds were

harvested for the subsequent field assays.

For comparing the suitability of the improved anther protocol, several parameters were analysed in the four  $F_2$  rice genotypes tested and NRCV980385 cultivar used in Serrat et al. (2014). These were: *callus* induction percentage (CI%) = number of anthers producing *calli*/number of plated anthers  $\times 100$ ; *callus* production ratio ( $CP_{\text{ratio}}$ ) = number of produced *calli*/number of anthers producing *calli*; green plant percentage (GR%) = number of green plant regenerated/number of transferred *calli*  $\times 100$ ; green double haploid plant percentage (GRDH%) = number of green DH plants regenerated/number of transferred *calli*  $\times 100$ . For comparing data among rice genotypes, we used two approximations: (i) visually, we calculated the confidence intervals (CI) using the following formula

$$CI = \% \pm 1.96 \times \sqrt{\frac{\% \times (1 - \%)}{\text{number of observations}}}$$

and used them as a mean of standard error; and (ii) statistically, we performed a Chi-squared test with Yates correction (Zar 2010). No visual nor statistical approximations were used for  $CP_{\text{ratio}}$ , since due to its nature neither CI nor Chi-squared test with Yates correction were possible to calculate. Please note that due to the experimental procedure of the anther culture, we did not use replicates, thus total values of several parameters for each genotype assayed were used instead.

Finally, for testing rice genotypes with commercial interest, we performed a general field assay on 70 double haploid in order to screen overall diseases resistance and production estimates. Selected genotypes were assayed in small scale field assays in La Càmera experimental fields (Amposta, Tarragona, Spain). For this, two designs were used: (i) plant agronomical trait evaluation: 25 plants per genotype assayed were planted in row as to have 20 cm between each plant and 50 cm between rows; (ii) plant production evaluation: 80 plants per genotype assayed were planted in row as to have 20 cm between each plant and 25 cm between rows. Plant agronomical traits such as plant height (i.e., from the base of the plant to the top of the panicle), susceptibility to rice stem borers and

Table 1. Parental cultivar (P1 and P2) and  $F_2$  rice genotypes produced by  $P1 \times P2$  cross are listed using the simplified code system according to La Càmera seed producer. Anther culture in vitro results are show: number of plated anthers, number of anthers producing *calli*, number of *calli* generated, *callus* induction percentage (CI,%), *callus* production ratio ( $CP_{\text{ratio}}$ ) and number of green double haploid plants regenerated in the four rice genotypes assayed. The error for CI(%) corresponds to the confidence interval.

Parental cultivar 1 (P1) <sup>a</sup>	Parental cultivar 2 (P2) <sup>a</sup>	$F_2$ rice genotype ( $P1 \times P2 \rightarrow F_1 \rightarrow F_2$ )	No of plated anthers	No of anther producing <i>calli</i>	No of produced <i>calli</i>	<i>Callus</i> induction (CI, %)	$CP_{\text{ratio}}$	No of green double haploid plants regenerated
rG3	NRCV980385 (rG0)	$F_2$ -30	20185	27	160	$0.133 \pm 0.050$	5.93	30
rG4	NRCV980385 (rG0)	$F_2$ -40	21301	99	547	$0.465 \pm 0.091$	5.53	70
rG4	rG2	$F_2$ -42	18880	37	152	$0.196 \pm 0.063$	4.11	7
rG4	rG5	$F_2$ -45	17456	72	360	$0.412 \pm 0.095$	5.00	17
—	—	NRVC980385 <sup>b</sup>	42660	4	66	$0.009 \pm 0.009$	16.50	29

<sup>a</sup>rG: Rice genotype. <sup>b</sup>Data for NRVC980385 was obtained from supplementary material in Serrat et al. (2014).

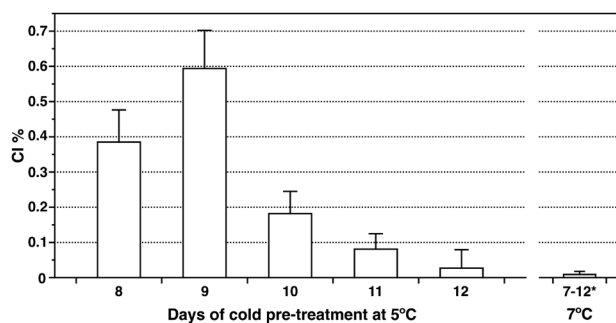


Figure 1. Effect of the cold pre-treatment on the callus induction percentage (CI,%) in the four rice lines assayed. The star (\*) data on the right side of the graph corresponds to NRVC980385 retrieved from supplementary material in Serrat et al. (2014), in which the cold pre-treatment was performed at 7°C during 7–12 days. Error bars correspond to the confidence intervals.

resistance to blast and brown spot (Mew and Gonzales 2002), number of spikes per plant and inter-homogeneity (homogeneity between plants of the same genotype) were recorded 120 days after sowing. For plant production traits, the parameters evaluated were humidity (%) at the time of data recollection, 1,000-grains weight, percentage of whole grains (unshattered milled grains/total milled grains $\times$ 100) and yield (kg of grains per hectare, kg/ha). NRVC980385 was used as a control to monitor field behaviour as it is a parental cultivar for F<sub>2</sub>-30 and F<sub>2</sub>-40, and is also common variety cultivated in the region (Català et al. 2007; Serrat et al. 2014).

Results of the cold-pretreatment duration test at 5.0 $\pm$ 0.1°C as well as that performed at 7°C by Serrat et al. 2014 is shown in Figure 1. It was observed that for the *callus* induction (CI, %), there were significant differences between the duration in days of the cold-pretreatment ( $\chi^2_{\text{Yates}}(4)=94.0699$ ,  $p<0.0001$ ; Figure 1), being 9 days the optimum for anther-derived *callus* induction. Moreover, it was also observed that a cold-pretreatment at 5°C during 9 days instead of 7°C during 7–12 days had a higher CI% in all the days tested, being 0.254 $\pm$ 0.072 in average and 0.009 $\pm$ 0.009, respectively. Our results differ to those of Kaushal et al. (2014b) where the optimum is for 5 days at 12°C. These contrasting results can be explained by the fact that Kaushal et al. (2014b) used *indica* varieties, whereas the ones used in our experiment are Mediterranean temperate *japonica* genotypes. Trejo-Tapia et al. (2002a, 2002b) also studied the effect of cold-pretreatment in anther-derived *callus* induction, but at 4°C. In their first study, 14 days was the best for the majority of the cultivars (tropical *japonica* sub-species) (Trejo-Tapia et al. 2002a), whereas in their second study 7 days was the best for H2500 cultivar (tropical *japonica* sub-species) (Trejo-Tapia et al. 2002b). Our cold-pretreatment duration is situated between both works, suggesting that 9 days is an ideal time for this stage of the anther culture procedure to enhance the *callus* production in Mediterranean temperate rice

*japonica* varieties. Moreover, the cold-pretreatment at 5°C during 9 days radically increases the CI%, as the lowest CI% value reported in this study is almost 15 times higher than the one reported with NRVC980385 (Serrat et al. 2014).

Regarding the anther culture procedure for obtaining new rice lines, an average of approximately 19,500 $\pm$ 1,600 anthers was plated for each of the four F<sub>2</sub> rice genotypes used in this experiment (details for each genotype in Table 1). *Calli* were produced from all of the four assessed genotypes differing in the CI%, but in average was higher than the one reported by Serrat et al. (2014). More in detail, it was observed that the genotype F<sub>2</sub>-30, the one with the lowest CI%, had an anther-derived *callus* induction 12 times greater than NRVC980385 (Table 1). On the other hand, genotypes F<sub>2</sub>-40 and F<sub>2</sub>-45 displayed a CI% 51.7 and 45.8 times higher, respectively, when compared to NRVC980385. It is worth noting that the three of the F<sub>2</sub> genotypes in which the cultivar 4 was one of the used parental, yielded the highest CI%, being the higher the genotype F<sub>2</sub>-40, cross between cultivar 4 and NRVC980385, with 0.465%. Statistical analysis showed significant differences between all the five rice genotypes (four F<sub>2</sub> rice genotypes and NRVC980385 cultivar) ( $\chi^2_{\text{Yates}}(4)=193.9229$ ,  $p<0.0001$ ). In the literature, CI% vary between as low as 0.2% to up to 77.9%, being the genotype the most important factor that determines these percentages (Bishnoi et al. 2000; Herath et al. 2007; Kaushal et al. 2014a, 2014b; Shahnewaz et al. 2004). Our CI% is situated in the lower ones, and it is probably due to the genotype of our F<sub>2</sub> rice genotypes, which do not favour *callus* formation as also observed in the study performed by Serrat et al. 2014. Several studies support this affirmation, since most of the differences can be explained by the genotype factor (Herath et al. 2007; Kaushal et al. 2014a; Khanna and Raina 1998; Shahnewaz et al. 2004; Yan et al. 1996). Despite the low CI% reported in this study when compared to the majority of the literature, the number of *callus* obtained was higher than that reported by other authors (Shahnewaz et al. 2003, 2004).

The higher anther-derived *callus* induction translated in a higher number of *calli*, 1,219 in total for the four F<sub>2</sub> genotypes assayed (Table 1). Moreover, the number of *calli* produced was much higher in this study compared to NRVC980385. The *callus* production ratio (CP<sub>ratio</sub>) was similar in the four rice genotypes, being F<sub>2</sub>-30 and F<sub>2</sub>-40 the ones that showed higher values of 5.9 and 5.5, respectively. In average, the CP<sub>ratio</sub> only corresponded to 31.2% of the displayed by NRVC980385 (Table 1) though we expected, as seen for CI%, that this parameter would be higher. This can be explained by considering the following: (i) average of anthers producing *callus* for our experiment was 59 $\pm$ 17 whereas for Serrat et al. (2014) only four anthers were used for *callus* production; (ii)

average of *callus* produced were  $305 \pm 94$  and 66 in our experiment and in Serrat et al. (2014), respectively. Thus, although having in average a low  $CP_{ratio}$  in our study, we expect to have a higher chromosomal variability of the rice genotypes since an elevated number of *calli* coming from a larger number of anthers was obtained. It is worth nothing that not much data is available in the literature regarding  $CP_{ratio}$  which in turn does not allow for much comparison. Nevertheless, it is of high importance since it gives information if regenerated plants come from several or few *calli*, thus we propose that this ratio should be regularly given.

The green plant percentage (GR,%) was in average  $89.6 \pm 5.9\%$  among the four rice genotypes tested as seen in Figure 2, which is similar to our prior results using NRVC980385. But, it is noteworthy that the GR% was considerably higher than in other articles (ranging from 2 to even 16 times more) (Herath et al. 2007; Kaushal et al. 2014a, 2014b; Shahnewaz et al. 2003, 2004; Trejo-Tapia et al. 2002a, 2002b). Statistical analysis also showed that there are significant differences between the 5 rice genotypes ( $\chi^2_{Yates}(4) = 6.3447$ ,  $p = 0.1478$ ; Figure 2).

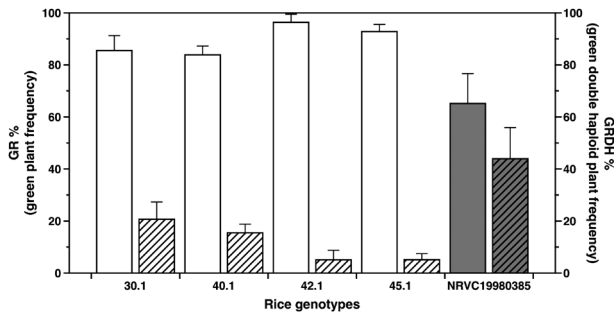


Figure 2. Total percentage of green regenerated plants (GR%) and total percentage of green double haploid regenerated plants (GRDH%) in the four rice lines assayed (white bars). Data for NRVC980385 was retrieved from supplementary material in Serrat et al. (2014) (grey bars). Solid bars correspond to GR% and hatched bars to GRDH%. Error bars correspond to the confidence intervals.

The green double haploid plant percentage (GRDH,%) reported in this study ranges from 11.5 to 47.1% of that reported with NRVC980385, being the genotypes F<sub>2</sub>-30 and F<sub>2</sub>-40 the ones displaying the higher GRDH% among our four F<sub>2</sub> rice genotypes (20.7 and 15.5%, respectively). In this study, more than 75 plants for each genotype were analysed, whereas in Serrat et al. (2014) only 43 in total were analysed. The GRDH% was significantly different among the 5 varieties ( $\chi^2_{Yates}(4) = 67.6942$ ,  $p < 0.0001$ ; Figure 2). This parameter is of the uttermost importance since those plants are viable and suited for field assay evaluations, which is the final purpose of this procedure. No data for this is available in the literature thus we cannot further compare. The last stage of the process was to acclimatize in vitro plants to greenhouse conditions in rice substrate. We transplanted a total of 547 green double haploid plants produced from the 4 F<sub>2</sub> genotypes, of which in average  $89 \pm 4\%$  were successfully grown to maturity (data not shown), which is greater than the  $67 \pm 8\%$  in average that is reported by Herath et al. (2007). Similarly, only  $12 \pm 8\%$  of the total of the transplanted plants showed more than 5% of sterility (data not shown), which is a better success rate than  $24 \pm 31\%$ , value observed in the work by Herath et al. (2007).

During the general field assay of 70 double haploid (DH) lines, it was observed that all DH plants coming from F<sub>2</sub>-42 and F<sub>2</sub>-45 along with some DH plants of F<sub>2</sub>-30 and F<sub>2</sub>-40 lacked agronomic and commercial interest (data not shown). Therefore, a total of 9 lines were selected for agronomical and production traits, which showed high inter-homogeneity and a high tillering activity (more than 40 tillers per plant; data not shown). The average height of the lines was  $70.7 \pm 2.2$  cm, which is in the range of those cultivated in the Ebro Delta. Moreover, 7 of them were shorter than Gleva, the shortest cultivated variety in the region (Pla et al. 2017). Overall, all evaluated lines showed a high

Table 2. Agronomical and production traits evaluation for the lines assayed in the field assays.

Anther-derived rice line	Height (cm) <sup>a</sup>	Plant agronomical traits evaluation			Plant production traits evaluation <sup>d</sup>			
		Fungal disease resistance <sup>b</sup>		Rice stem borers resistance <sup>b,c</sup>	Humidity (%)	1,000-grain weight (g)	Whole grains (%)	Yield (kg/ha)
		Blast <sup>c</sup>	Brown spot <sup>c</sup>					
F <sub>2</sub> -30.C1	68.5 ± 1.9	+++	+++	++	13.8	27.5	67.3	5740
F <sub>2</sub> -30.C2	70.5 ± 0.6	+++	+++	++	14.0	28.8	66.7	5828
F <sub>2</sub> -40.C15	66.3 ± 4.1	+++	+++	++	13.9	32.5	67.8	11393
F <sub>2</sub> -40.D37	68.5 ± 1.3	+++	+++	+	13.8	35.0	63.3	11598
F <sub>2</sub> -40.D39	62.8 ± 2.1	+++	+++	+++	13.6	35.0	61.4	10338
F <sub>2</sub> -40.D118	65.3 ± 0.5	+++	+++	+	13.8	35.0	66.9	11328
F <sub>2</sub> -40.D173	59.0 ± 2.7	+++	+++	++	13.9	30.0	63.0	11670
F <sub>2</sub> -40.D174	61.5 ± 1.3	+++	+++	++	13.9	32.5	65.9	11760
F <sub>2</sub> -40.D266	90.3 ± 4.4	+++	+	-	14.1	35.0	63.9	13553
NRVC980385	94.0 ± 2.7	+++	+++	+	14.2	27.5	65.3	11325

<sup>a</sup> The value shown correspond to the mean of 25 plants and the SD. <sup>b</sup> Resistance scale is the following: -: sensible; +: low resistance; ++: medium resistance; +++: high resistance. <sup>c</sup> Blast and brown spot diseases are caused by *Magnaporthe oryzae* and *Helminthosporium* sp. respectively, and rice stem borers by *Chillo suppressalis*. <sup>d</sup> Production traits evaluation data for NRVC980385 was recorded at the same time of the F<sub>2</sub> rice lines assayed.



resistance to fungal diseases and medium resistance to rice stem borers (Table 2), except for F<sub>2</sub>-40.D266 plants which was promising in terms of production during the general field assay. Regarding blast resistance, it was in general higher in comparison to the varieties regularly cultivated in the region of the Ebro Delta (Català et al. 2009; Pla et al. 2017). No literature was available for comparing tolerance to brown spot disease and the rice stem borers in local field conditions. Despite this, resistance to brown spot was similar to that of the control variety (NRVC980385), and rice stem borers resistance was higher than NRVC980385 for several lines. The 1,000-grain weight (determined by grain length, width and thickness) of the lines was in average 31.6g, comparable among them since the humidity range was 13.6–14.2%. Of the assayed lines, all of them with the exception of F<sub>2</sub>-30.C1, as seen in Table 2, showed a 1,000-grain weight higher than NRVC980385 and other *indica* and *japonica* varieties (Fan et al. 2006; Koutroubas and Ntanos 2003). The whole grains percentage was variable among the 9 lines assayed but ranged between 60–70%, similar to NRVC980385 and several other Spanish varieties values (Català et al. 2009), thus these lines are suitable for large scale production. In terms of yield, lines of the F<sub>2</sub>-30 genotype were below those reported for Gleva (most cultivated variety in the region) and NRVC980385 cultivar (Pla et al. 2017), and half of the values reported for F<sub>2</sub>-40 genotype lines (Table 2). On the other hand, lines of the F<sub>2</sub>-40 genotype displayed higher yields than Gleva (Pla et al. 2017). Furthermore, the observation that F<sub>2</sub>-40.D266 was promising in terms of production was certain as its yield was the highest among the lines tested and higher than the Spanish rice varieties including the NRVC980385 cultivar (Pla et al. 2017). Nevertheless, to fully characterize and evaluate the assayed lines, direct seeded field assays should be performed in a medium (and maybe even large scale) in order to better assess for pathogens resistance, plant height and yield.

In conclusion, we have shown that the improved anther culture protocol can be successfully applied in different F<sub>2</sub> rice genotypes between temperate *japonica* rice genotypes to obtain green double haploid plants. Moreover, we have observed that genotype is one of the main factors that affects the anther culture protocol success. Despite this, we have determined that the cold-pretreatment improvement, 9 days at 5.0±0.1°C, greatly increases the anther-derived *callus* induction in temperate *japonica* Mediterranean rice crossed genotypes at the F<sub>2</sub> generation, since the number of green double haploid plants obtained at the end of the anther culture procedure was high. Furthermore, 7 of the 9 lines evaluated in the field showed good qualities at the agricultural and production level. Therefore, these varieties are suited to be submitted to direct

seeded medium scale assays before registry for their subsequent commercialization. Thus, in conclusion, our proposed method for Mediterranean *japonica* rice is highly applicable to rice genotypes at the F<sub>2</sub> generation of different *japonica* rice cultivars for producing new lines that could be registered and commercialized as new varieties.

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### Conflicts of interest

The authors declare that they have no competing interests.

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