

Colchicine and osmotic stress for improving anther culture efficiency on long grain temperate and tropical japonica rice genotypes

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Abstract Anther culture is a fast tool to obtain double haploid plant lines for breeding purposes. In rice, this procedure is commonly performed in two steps: i) induction of calli from anthers and ii) regeneration of plantlets from calli. It has been stated that genotype highly influences the anther culture efficiency, so the media used in each step should be optimized for each variety. In this study, we tested different media modifications of an efficient protocol optimized for a medium sized grain temperate japonica NRVC980385, used as a control, in a long grain temperate japonica rice variety (NRVC20120346), and two long grain tropical japonica varieties (303012 and 303013).

We found that the addition of 150 mg l⁻¹ colchicine to the induction medium worked best for all genotypes except for NRVC20120346, whose best induction was obtained with the colchicine-free medium. Referring to regeneration, increased gelling agent in the medium provided the best rates in NRVC980385, improving our former NRVC980385-optimized anther culture protocol. Sorbitol fortified regeneration medium worked the best in the case of the long grain varieties. The presence of colchicine in the induction medium was also related to a higher obtention of double haploid plantlets. This study highlights that genotype is a key factor in the performance of rice anther culture. It has set a first anther culture study on long grain japonica varieties and optimizes the anther culture protocol for temperate japonica medium grain NRVC980385 with the use of colchicine and other additives that increase osmotic stress.

Key words: anther culture, colchicine, double haploid, japonica rice, *Oryza sativa*, osmotic stress.

Rice (*Oryza sativa* L.) is one of the most important crops worldwide feeding more than three billion people daily, providing up to 25% of the caloric needs (Birla et al. 2017). Its production in 2017 reached 503.9 million tones (milled equivalent) and is expected to increase with human food use driving its growth (FAO 2018). This increasing rice demand, in addition to the biotic and abiotic stresses that rice is exposed to, makes necessary an increment of yield (Mammadov et al. 2018). Breeding techniques allow to obtain new rice varieties or improve the existing ones by incorporating desirable traits. Conventional breeding is time consuming, and the stabilization of the new lines from an F1 cross can extend to 8 years (Germanà 2011; López-Cristoffanini et al. 2018). The anther culture is a biotechnological tool first developed in rice by Niizeki and Oono (1968) that allows to generate homozygous lines (double haploids) from any diploid segregating line that might fix interesting breeding characters (Serrat et al. 2014). The whole anther culture process takes up approximately

one year, reducing drastically the classical breeding cycle (Hooghvorst et al. 2018).

The anther culture is a two-step technique that allows in rice to force haploid microspores to develop under a sporophytic pathway by using growth regulators. These microspores induce calli, which are put in regeneration medium in order to develop plantlets (Mishra and Rao 2016). The plant material genotype highly determines the efficiency of anther culture protocols, therefore these are currently optimized for each rice line. Media additives such as growth regulators, involved in embryogenesis, antimitotic agents as well as amino acids, which are related to the degeneration of wall tissues (Maheshwari et al. 1982), have shown to improve the calli induction and regeneration rates (Germanà 2011). It has been proved that the presence of colchicine, an antimitotic agent, in the calli induction medium may increase the amount of regenerated double haploid green plantlets in some plant crops such as maize (Obert and Barnabas 2004), triticale (Ślusarkiewicz-Jarzina et al. 2017) or rice (Alemanno and

Guiderdoni 1994). Furthermore, stresses can also help improving green double haploid plantlet regeneration (Germanà 2011), being osmotic stress one of them. It allows a greater number of cells to differentiate, since it physiologically isolates the pre-embryonic cells by disrupting the plasmodesmatal connections between them (Wetherell 1984). As osmotic stress, there are two methods for adding sorbitol or high concentration gelling agents to the medium. The presence of high amounts of gelling agents helps to impose this stress, being believed that Gelrite is the most suitable since it does not release impurities to the medium (Calleberg and Johansson 1996). Actually, Tsukahara and Hirosawa (1992) found that the addition of sorbitol or high concentration of gelling agents promoted plant regeneration from rice (*O. sativa* L. cv. sasanishiki) callus.

The aim of this study is to adapt an anther culture protocol already improved for NRVC980385 and other medium grain Mediterranean temperate japonica cultivars (Hooghvorst et al. 2018; López-Cristoffanini et al. 2018; Serrat et al. 2014), to be used for tropical and temperate japonica long grain varieties. For this, we assayed (i) a colchicine-supplemented induction medium, and (ii) three regeneration media with different osmotic potential to improve plantlet regeneration. The rice genotypes used in this research were four Mediterranean cultivars (*O. sativa* ssp. *japonica*). Two long grain tropical japonica cultivars coded as 303012 and 303013, one long grain temperate japonica coded as NRVC20120346 and a medium grain temperate japonica coded as NRVC980385. All the seeds were provided by Càmarà Arroçera del Montsià SCCL (Amposta, Tarragona, Spain).

In order to perform this study, the plant material was cultivated in controlled greenhouse conditions at the Servei de Camps Experimentals (Universitat de Barcelona). Tillers at the booting stage were collected and disinfected as described by Serrat et al. (2014). Tiller cold pretreatment was carried out as suggested by Hooghvorst et al. (2018). Afterwards, tillers were disinfected again using ethanol 70% for one minute followed by one rinse with sterile distilled water for another minute in sterile conditions. The spikelets were extracted from the foliar sheaths and selected following the criteria of Afza et al. (2000). The selected spikelets were submitted to a third disinfection, and the anthers were plated in calli induction media as described by Serrat et al. (2014). Plates were incubated during 6–8 weeks in darkness at 24°C.

Two different calli induction media were assayed in this work: (i) a colchicine-free medium coded as 0D1 (Serrat et al. 2014) and (ii) a 150 mg l⁻¹ colchicine-supplemented medium coded as 150D1 (Hooghvorst et al. 2018). The anthers sown in 150D1 were transferred to 0D1 after 24 h. The anthers were maintained in the

0D1 induction medium for a maximum of three months with a two-week medium change. When a callus of ca. 2 mm in diameter emerged from a microspore, it was transferred to a regeneration medium. Three different plantlet regeneration media were assayed in this step: (i) a control regeneration medium named XACRM, previously defined and used by Serrat et al. (2014), (ii) a sorbitol-supplemented XACRM medium (which was named SARM) and (iii) a Gelrite-enriched XACRM medium (which was named GERM). The control XACRM medium was composed by 3.957 g l⁻¹ N6 salts & vitamins (Chu 1981), 30 g l⁻¹ sucrose, 0.25 g l⁻¹ L-proline, 1 g l⁻¹ casein hydrolysate, 1 mg l⁻¹ naphthaleneacetic acid, 2 mg l⁻¹ kinetin, 0.5 g l⁻¹ 2-(*N*-morpholino) ethanesulfonic acid (MES) and 3 g l⁻¹ Gelrite. SARM was supplemented with 20 g l⁻¹ sorbitol and GERM 4.5 g l⁻¹ Gelrite. Finally, the pH was adjusted to 5.8 in all media. Every four weeks, calli were transferred to the corresponding fresh regeneration medium for up to three months. Green little plantlet masses regenerated from calli were transferred to hormone-free MS based medium (Murashige and Skoog 1962) described by Serrat et al. (2014) known as root medium (RM). All regenerated albino plantlets were discarded in this study. A plantlet propagation was carried out 21 days after the transference to RM tubes to avoid any chimeric plantlet having two or more genotypes or ploidy levels. Ploidy analyses were performed for each separate plantlet by flow cytometry as described by Hooghvorst et al. (2018).

To determine the efficiency of the different anther culture steps, some parameters were calculated for each genotype, such as the percentage of calli induction (number of anthers producing calli · 100 · number of sown anthers⁻¹), plantlet regeneration (plantlets regenerated · 100 · number of total calli⁻¹), percentage of green plantlets (number of green plantlets · 100 · number of induced calli⁻¹) and percentage of green double haploid plantlets (number of green double haploid plantlets · 100 · number of green plantlets⁻¹). To determine significant differences in the first two parameters, a chi-squared test (χ^2 test) was assayed with 95% of confidence.

Concerning to the first step of the anther culture protocol, the number of sown anthers and calli induction (CI%) of the four genotypes according to the presence of colchicine are listed in Table 1. Statistical analysis showed that there were significant differences in calli induction in relation to the media ($p < 0.05$). In the case of long grain temperate japonica rice genotype, NRVC20120346, the induction rate in 150D1 medium was reduced compared to 0D1 medium. However, in the case of tropical japonica genotypes, 303012 and 303013, which were tested for the first time, induction rates were higher in 150D1 medium than in 0D1 medium, although for all cases induction rates were less than 1%. Besides, the NRVC980385 cultivar induced calli 20-fold more in

150D1 than in 0D1 medium. This result is very similar to the one obtained by Hooghvorst et al. (2018), performed under the same conditions and cultivar, with a 7.39% induction rate. This proves again the high influence of genotype in the anther culture efficiency. An explanation for the varying induction percentages could be that

temperate japonica and tropical japonica varieties are genetically divergent in spite of belonging to the same japonica sub-species (Kovach et al. 2007).

Referring to the second step of the anther culture protocol, different plantlet regeneration percentages were obtained depending on the three tested media. This is reflected in Table 2. Statistical analysis proved significant differences in plantlet regeneration depending on the regeneration media ($p < 0.05$). In general, the calli coming from 150D1 were the ones that regenerated plantlets, and those induced in 0D1 media did not regenerate any, with the exception of NRVC20120346 in SARM regeneration media. This might be due to the low number of calli induced and therefore regenerating, and also the fact that colchicine improves regeneration rates (Chen et al. 2001). Focusing on the regeneration media, SARM was the only medium in which calli regenerated in all genotypes, although the rates were lower for the tropical japonica genotypes than for the temperate japonica ones. Moreover, SARM medium was the one that provided the highest regeneration percentage in all

Table 1. Anther culture parameters of the calli induction step.

| Genotype | Calli induction medium | No. of sown anthers | No. of anthers producing calli (%) |
|--------------|------------------------|---------------------|------------------------------------|
| NRVC980385 | 0D1 | 1698 | 6 (0.35) |
| | 150D1 | 4331 | 328 (7.57) |
| NRVC20120346 | 0D1 | 857 | 37 (4.32) |
| | 150D1 | 3613 | 34 (0.94) |
| 303012 | 0D1 | 2608 | 3 (0.12) |
| | 150D1 | 8258 | 39 (0.47) |
| 303013 | 0D1 | 2220 | 3 (0.14) |
| | 150D1 | 6634 | 32 (0.49) |

Table 2. Percentage of calli induction, number of regenerated plantlets and percentage of green double haploid plantlets obtained according to the calli induction media (0D1, 150D1) and the regeneration media (XACRM, GERM, SARM).

| Genotype | Regeneration medium | Origin of calli | No. of cultured calli | No. of calli regenerating plantlets (%) | No. of regenerated plantlets | No. of green double haploid plantlets (%) |
|--------------|---------------------|-----------------|-----------------------|---|------------------------------|---|
| NRVC980385 | XACRM | 0D1 | 0 | — | 0 | — |
| | | 150D1 | 111 | 52 (46.85) | 176 | 51 (28.98) |
| | SARM | 0D1 | 2 | 0 (0.00) | 0 | — |
| | | 150D1 | 108 | 70 (64.81) | 439 | 173 (39.41) |
| | GERM | 0D1 | 4 | 0 (0.00) | 0 | — |
| | | 150D1 | 109 | 77 (70.64) | 219 | 89 (40.64) |
| NRVC20120346 | XACRM | 0D1 | 11 | 0 (0.00) | 0 | — |
| | | 150D1 | 14 | 6 (42.86) | 16 | 0 (0.00) |
| | SARM | 0D1 | 15 | 2 (13.33) | 24 | 0 (0.00) |
| | | 150D1 | 10 | 6 (60.00) | 0 | — |
| | GERM | 0D1 | 11 | 0 (0.00) | 1 | 0 (0.00) |
| | | 150D1 | 10 | 6 (60.00) | 31 | 0 (0.00) |
| 303012 | XACRM | 0D1 | 1 | 0 (0.00) | 0 | — |
| | | 150D1 | 11 | 0 (0.00) | 0 | — |
| | SARM | 0D1 | 0 | — | 0 | — |
| | | 150D1 | 12 | 2 (16.67) | 23 | 7 (30.43) |
| | GERM | 0D1 | 2 | 0 (0.00) | 0 | — |
| | | 150D1 | 16 | 0 (0.00) | 0 | — |
| 303013 | XACRM | 0D1 | 1 | 0 (0.00) | 0 | — |
| | | 150D1 | 11 | 1 (9.09) | 0 | — |
| | SARM | 0D1 | 2 | 0 (0.00) | 0 | — |
| | | 150D1 | 8 | 2 (25.00) | 0 | — |
| | GERM | 0D1 | 0 | — | 0 | — |
| | | 150D1 | 13 | 2 (15.38) | 0 | — |

three long grain genotypes. GERM medium caused lower rates of regeneration than SARM in NRVC20120346 and 303013, and no regeneration at all in 303012 genotype. Globally, NRVC980385 was the genotype that presented the highest percentage of plantlet regeneration. Also, it was the genotype that better responded to any of the three different regeneration media, being GERM the one that regenerated the most plantlets. Yoshida et al. (1994) tried different combinations of sorbitol percentage and hormone concentration in the regeneration medium of two japonica rice cultivars. It would be interesting to carry out an essay of SARM with different hormone and sorbitol concentrations to see the improvement it would have on regeneration rates.

The regenerated plantlets were submitted to a cytometry analysis. The percentage of double haploid green plants of each genotype and for each induction and regeneration medium is listed in Table 2. In the case of the temperate japonica genotypes, no green double haploid plantlets were obtained for the NRVC20120346 genotype. NRVC980385 produced green double haploid plantlets from the calli originated in the 150D1 medium in all regeneration media. However, no green double haploid plantlets were regenerated from calli coming from the 0D1 medium, which is not hazardous since colchicine favors the green double haploid production (Chen et al. 2001). For the 303012 tropical japonica genotype, green double haploid plantlets were only obtained in SARM regeneration medium from the calli induced in 150D1.

NRVC980385 and 303012 were the only genotypes that regenerated green double haploid plantlets from the calli induced the colchicine-supplemented medium. Alemanno and Guiderdoni (1994) described a 1.5- to 2.5-fold increase in double haploid green plantlets production by using a colchicine-supplemented medium in the calli induction step. Since no diploidization occurred in NRVC20120346 during the anther culture steps, diploidization of haploid green plantlets could be forced with antimitotic agents *in vitro*. Hooghvorst et al. (2018) performed diploidization in haploid plantlets proceeding from the anther culture of the temperate japonica variety NRVC980385, improving the efficiency of their anther culture experiment. They found that 250 mg l⁻¹ of colchicine or 1.25 mg l⁻¹ of oryzalin treatments provided the best diploidization rates.

This study has shown the importance of genotype in the performance of rice anther culture in combination with medium additives. It has not only improved the previous anther culture protocol for NRVC980385 cultivar, but it has also performed for the first time an essay on long grain tropical and temperate varieties. Besides, it has proved that colchicine enhances the anther culture at both induction and regeneration steps, and the usefulness of osmotic stress for plantlet regeneration.

This work contributes to the knowledge in japonica rice varieties anther culture, since few studies are yet published. Further investigations will help to elucidate the differential characteristics that anther culture of japonica rice varieties presents, meaning a substantial improvement in the obtaining of double haploids, and therefore, in breeding.

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Author contribution

Irene Ferreres: design of the study, reporting of the study and *in vitro* experiments. Mirari Ortega: *in vitro* experiments and reporting of the study. Camilo López-Cristoffanini: *in vitro* experiments and proofreading. Salvador Nogués: design of the study and proofreading. Xavier Serrat: proofreading and *in vitro* experiments.

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