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ORIGINAL ARTICLE

Antimitotic and hormone effects on green double haploid plant production through anther culture of Mediterranean japonica rice

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Abstract

Rice double haploid (DH) plants are produced mainly through anther culture. In order to improve the anther culture protocol, microspores of two japonica rice genotypes (NRVC980385 and H28) were subjected to three growth regulator combinations and four colchicine treatments on induction medium. In addition, a post anther culture procedure using colchicine or oryzalin was tested to induce double haploid plantlets from haploid plantlets. A cold pre-treatment of microspores for 9 days at 10 °C increased callus induction 50-fold in the NRCV980385 genotype. For both genotypes, 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin on colchicine-free induction medium gave the best culture responses. The culturability of both genotypes changed on colchicine-supplemented induction media. A high genotype dependency was recorded for callus induction, callus regenerating green plantlets and regeneration of green double haploid plantlets. Colchicine at 300 mg L^{-1} for 48 h enhanced callus induction 100-fold in H28. Colchicine-supplemented media clearly improved green double haploid plantlet regeneration. We showed that the post-anther culture treatment of haploid plantlets at 500 mg L^{-1} of colchicine permitted fertile double haploid plantlets to be generated. Finally, an enhanced medium-throughput flow cytometry protocol for rice was tested to analyse all the plantlets from anther and post anther culture.

Keywords Mediterranean japonica rice · Anther culture · Hormones · Colchicine · Antimitotics · Double haploid

Introduction

Doubled haploid lines (DHs) are produced when spontaneous or induced chromosome duplication of haploid cells occurs. DH plants are complete homozygous individuals that can be produced within a year through anther or microspore culture. Therefore, the production of homozygous lines from heterozygous parents is feasible and shortens the time required to obtain them (Germanà 2011). Nowadays, anther culture is being used to produce DH plants in more than 250 species, including major cereals such as rice, wheat, maize, barley and also economically important trees, fruit crops and medicinal plants (Maluszynska 2003).

Rice DH plant production is mainly obtained through anther culture. Niizeki and Oono (1968) were the first to produce haploid rice plantlets through anther culture. Rice anther culture is a two-step process with initial callus development and subsequent green plantlet regeneration from embryogenic callus (Mishra and Rao 2016). Since the first report of anther culture, much research has aimed at optimizing the media used at each step in the process to enhance callus induction and callus regeneration (Herath et al. 2010; Pauk et al. 2009). This work has focused on overcoming limiting factors that reduce the efficiency of green DH plantlet production such as high genotypic dependency, low frequency of callus induction and plantlet regeneration, the low percentage of doubled haploids

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produced and the high ratio of albino plantlets (Lentini et al. 1997). The application of stress during the developmental period of pollen grains, osmotic stress applied to cells during culture, the composition of the culture media, and the addition of antimitotic agents, gelling agents or growth regulators amongst many exogenous factors may affect the success of anther culture in rice (Mishra and Rao 2016). Endogenous factors such as the rice variety and genotype also affect anther culture success. Indica rice varieties have a limited response to anther culture due to early necrosis, poor callus proliferation and a high regeneration of albino plantlets (Chen et al. 1991), unlike japonica varieties where green DH plant production is more efficient (He et al. 2006).

Despite the improvements and progress achieved in every step of the anther culture procedure, there is still a need to optimize conditions for higher rates of green DH plant production while reducing the amount of work in each step. Colchicine is an antimitotic compound widely used in microspore culture and has been shown to improve results in terms of green double haploid plant production (Forster et al. 2007) in maize (Obert and Barnabás 2004), barley (Thompson et al. 1991), wheat (Barnabás et al. 1991), rapeseed (Weber et al. 2005), and other species. However, few authors have reported the use of colchicine in rice anther culture. Alemanno and Guiderdoni (1994) were the first to study a routine in vitro colchicine treatment to increase DH plant production in rice. In addition, post anther culture procedures have rarely been used in green haploid plantlets regenerated from anther culture. Finally, such a procedure can be undertaken either in vivo by treating tillers with antimitotic compounds such as colchicine in order to increase the DH recovery from haploid plantlets (Jensen 1974; Zapata-Arias 2003; Chen et al. 2002) or in vitro as explained in this work.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-*D*) and naphthaleneacetic acid (NAA) are the most used hormones for rice callus induction from anthers (Trejo-Tapia et al. 2002).

The aim of this study is to improve the anther culture efficiency in two japonica rice genotypes by assessing some factors that could improve the numbers of green double haploid plants. Thus we tested (i) the effect of different growth regulators (2,4-*D*, NAA and Kinetin) in the anther culture induction medium, (ii) the effect of different colchicine doses in the anther culture induction medium and (iii) a post anther culture procedure to increase plant DH production from haploid plantlets through colchicine and oryzalin in vitro treatments. We showed that colchicine-supplemented media increase green plantlet double haploid production and 500 mg L^{-1} of colchicine in a post-anther culture procedure enabled recovery of green double haploid plantlets from haploid plantlets to be maximized.

Materials and methods

Plant material and growth conditions

The commercial temperate japonica rice variety NRVC980385 and a temperate japonica F2 hybrid called H28, provided by the *Càmara Arrossera del Montsià SCCL* cooperative, were used as plant material. Plants were grown in greenhouse conditions at the *Servei de Camps Experimentals* at the University of Barcelona (Barcelona, Spain) in 4 L plastic containers filled with rice substrate as previously described (Serrat et al. 2014).

Anther culture procedure

Tillers were selected at the booting stage, when the distance from the flag leaf to the auricle of the penultimate leaf was 5–12 cm. The time of collection was from 8:00 to 9:30 as recommended by Chen et al. (1991). Collected tillers were soaked in 70% ethanol for 1 min, rinsed twice with distilled water and were then cold pre-treated for 9 days at 10 °C in polystyrene bags, prior to being surface disinfected again as above. Tillers were dissected to obtain the panicles in a laminar flow cabinet. Panicles were soaked in 70% ethanol for 1 min, rinsed twice and soaked in 10% sodium hypochlorite solution with Tween 20 (30 drops L^{-1}) and 35% HCl (50 drops L^{-1}) for 3 min, and rinsed five times in sterile distilled water. Anthers were obtained from the panicles and plated into 90 mm petri dishes (Sterilin LTD, Cambridge). Basal induction medium consisted of Chu N6 modified as follows: N6 standard salts and vitamins fortified with a combination of growth regulators, 1 g L^{-1} casein enzymatic hydrolysate, 250 mg L^{-1} L-proline, 2 mg L^{-1} 500 mg L^{-1} 2-(*N*-morpholino) ethanesulfonic acid (MES), 30 g L^{-1} sucrose and 3 g L^{-1} Gelrite.

The anther culture procedure was carried out in parallel for all the combinations of different growth regulators and their concentrations, colchicine concentrations and colchicine exposure times (Table 1). Regarding growth regulators, three combinations were used: (i) treatment D1, 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; (ii) treatment D2, 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, as used by Serrat et al. (2014) and Chen et al. (2002); and (iii) treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin, as used by Alemanno and Guiderdoni (1994). Colchicine was assayed at 0 (control), 150 and 300 mg L^{-1}, each for the two exposure times of 24 and 48 h. Thus, five colchicine treatments were tested and named as concentration/exposure time: 0/0 (control), 150/24, 150/48, 300/24 and 300/48. Six petri dishes were sown with approximately 100 anthers

Table 1 Conditions for induction media assayed for both genotypes

Growth regulator			Colchicine treatment		Conditions
2,4-*D* (mg L^{-1})	NAA (mg L^{-1})	Kinetin (mg L^{-1})	Concentration (mg L^{-1})	Exposure time (h)	Concentration/time-hormone
1	0	1	0	0	0/0-D1
			150	24	150/24-D1
			150	48	150/48-D1
			300	24	300/24-D1
			300	48	300/48-D1
2	0	1	0	0	0/0-D2
			150	24	150/24-D2
			150	48	150/48-D2
			300	24	300/24-D2
			300	48	300/48-D2
0	2	0.5	0	0	0/0-NA
			150	24	150/24-NA
			150	48	150/48-NA
			300	24	300/24-NA
			300	48	300/48-NA

Three growth regulator treatments were tested: *D1* 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, *D2* 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; and treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin; and three colchicine treatments concentrations were applied, 0 (control condition), 150 and 300 mg L^{-1}

for each condition. After 24 or 48 h, anthers inoculated in colchicine-supplemented media were transferred to exactly the same medium but colchicine-free.

Anthers were kept in the dark at 24 °C and analysed weekly for 8 weeks. Microspore-derived calluses of 1–2 mm diameter that emerged from anthers were transferred to callus regeneration medium as described by Serrat et al. (2014). Anthers that induced callus were removed to ensure a count of one callus per anther, to avoid overestimation of callus induction and to match the number of calluses and induced calli for data analysis. Calluses were transferred to plantlet regeneration medium containing Chu N6 (Chu 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 mg L^{-1} sucrose and 3 g L^{-1} Gelrite. IWAKI 94 mm petri dishes (Asahi Techno Glass Corporation, Amagasaki) were filled with 25 mL of the medium. Calluses were transferred after 28 days onto fresh regeneration medium. Cultures were kept at 25 °C and illuminated with 50–70 µmol m^{-2} s^{-1} fluorescent light under a 16/8 h day/night photoperiod until plantlet formation occurred.

The tiny but fully formed albino and green plantlets (0.5-3 cm length) were transferred into tubes with hormone-free MS (Murashige and Skoog 1962) medium as described by Serrat et al. (2014). Subsequently, clearly sprouting individual plantlets were propagated under conditions as described for regeneration above.

All components of the media were supplied by Duchefa Biochemie BV (The Netherlands). Media were prepared using distilled water and the pH was adjusted to 5.7 by adding 1M KOH (Sigma-Aldrich Co). All components including growth regulators were added before standard autoclave sterilization (121 °C for 20 min).

Ploidy-level determination

The ploidy of green and albino regenerated plantlets was determined by flow cytometry following the procedure of Cousin et al. (2009) with slight modifications. About 5 mg of young leaves were collected and put into ice-cold 2 mL microcentrifuge tubes each with a single steel bead (3 mm diameter). To each tube, 300 µL of cold lysis buffer (0.1 M citric acid and 0.5% Triton X-100 in distilled water) were added. Tubes were cooled at − 20 °C for 10 min. Samples were shaken at 25 Hz for a total of 48 s in a MM 400 tissue lyser (Retsch, Haan, Germany). The aliquot from each tube was filtered through a 22 µm nylon filter (Sefar Maissa, Blacktown, Australia), gently vacuumed and transferred to a flow cytometry sample tube (Beckman Coulter Inc., Pasadena, California, USA). Afterwards, 150 µL of propidium iodide (PI) stain solution [0.25 mM Na_2HPO_4, 10 mL 10× stock (100 mM sodium citrate, 250 mM sodium sulfate) and 9 M PI made up to 100 mL with Milli-Q water] was added to each tube. Tubes were then sealed and kept on ice in the dark for 1 h before flow cytometry (FCM) analysis. The stained nuclei samples were analysed using a Gallios™ Flow Cytometer (Beckman Coulter Inc., Pasadena, California, USA) with a 488-nm laser at the Cytometry Unit (Scientific and Technological Centres, University of Barcelona) and a 32-well carrousel. One diploid control (NRVC 980385) sample was included every seven measurements. Samples analysed with a clearly defined peak as the reference ploidy control were classified as DH, whereas those producing half the fluorescence were classified as haploids. Flow cytometry data was analysed using Summit Software v4.3 (Beckman Coulter Inc., Pasadena, California, USA).

Diploidization of haploid green plantlets

Green haploid plantlets regenerated from anther culture were subjected to a post anther culture in vitro treatment with colchicine at 1000, 500 and 250 mg L^{-1}, or oryzalin at 5, 2.5 and 1.25 mg L^{-1}; both in a solution containing 1% DMSO and Tween 20 (4 drops·L^{-1}) in sterilized distilled water. Prior to the antimitotic treatment, plantlet stems and roots were trimmed to 3 cm in length and were incubated in the antimitotic solution for 5 h on a shaker at 120 rpm at 25 °C and maintained under sterile conditions in a laminar

flow cabinet. Thereafter, the plantlets were transferred to hormone-free MS medium as described before and by 3–4 weeks of growth plantlets that had survived and reached 10–15 cm in size were collected to perform flow cytometry analysis as described before.

Statistics

All parameters were divided by the number of anthers sown for each treatment and multiplied by 100 in order to obtain percentages: induced calluses (IC), number of calluses regenerating green plantlets (CRGP), number of calluses regenerating albino plantlets (CRALP), number of calluses regenerating green and albino plantlets (CRGAP), number of green plantlets regenerated (GPR), number of double haploid plantlets regenerated (DHPR) and number of green double haploid plantlets regenerated (GDHPR). The three concentrations of growth regulators in the colchicine-free media were compared with each other, and each colchicine treatment was also compared individually with the corresponding control medium (0/0) according to the growth regulator hormone concentration (D1, D2 and NA). Growth regulators and colchicine treatments were analysed separately for both genotypes. To determine significant differences between the conditions assayed, a Chi-Square ($P < 0.05$) test for homogeneity was used.

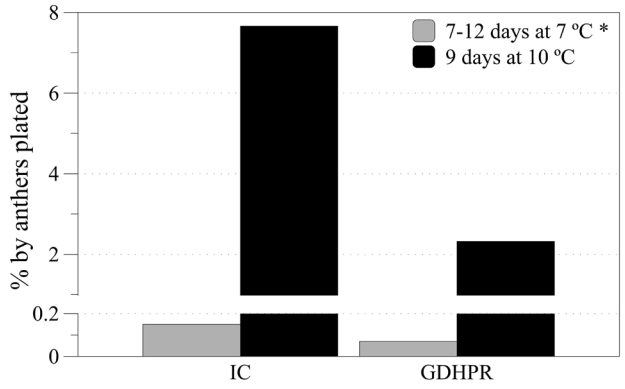

Fig. 1 Induced callus (IC) and green double haploid plantlets regenerated (GDHPR) for NRCV980385 in 0/0-D2 treatment with a cold pre-treatment of 7–12 days at 7 °C* and 9 days at 10 °C (this work). Reproduced with permission from Serrat et al. (2014)

Results

Effect of the cold pre-treatment on callus induction

The percentages of induced calluses (IC) and green double haploid plantlets regenerated (GDHPR) were compared according to the cold pre-treatment applied (Fig. 1) Cold pre-treatment was adjusted to 9 days at 10 °C for NRCV980385 (data not shown) as suggested by Serrat et al. (2014). Following cold pre-treatment for 9 days at 10 °C the IC and GDHPR percentages were 51 times and 33 times higher, respectively, than the 7–12 day pre-treatments at 7 °C.

Effect of different growth regulators on rice anther culture

The culturability of both genotypes with the different growth regulators in colchicine-free induction media is shown in Table 2. In D2 medium, the culturabilities of NRCV980385 and H28 were greater for all the parameters analysed.

For NRCV980385, there were no significant differences ($P > 0.05$) between the three growth regulator treatments (Table 2) for any of the anther culture parameters. Nevertheless, there was a tendency for D2 conditions (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin) to yield higher values than the other treatments for callus induction (IC), callus that regenerates green plantlets (CRGP), regenerated green plantlets (RGP), regenerated double haploid plantlets (RDHP) and regenerated green double haploid plantlets (RGDHP). The values of the D1 and NA growth hormone regulator

Table 2 Culturability results for the temperate japonica rice variety, NRVC980385, and the temperate japonica F2 hybrid, H28, among three growth regulator treatments assayed without colchicine: *D1* 1 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin, *D2* 2 mg L^{-1} 2,4-*D* and 1 mg L^{-1} kinetin; and treatment NA, 2 mg L^{-1} NAA and 0,5 mg L^{-1} kinetin

Rice genotype	Hormone combination	Anther culture parameters				
		IC	CRGP	GRP	DHPR	GDHPR
NRCV 980385	D1	6.05	0.36	1.96	0.36	1.60
	D2	7.66	0.89	3.21	0.89	2.32
	NA	6.96	0.17	1.39	0.17	1.22
H28	D1	0.18*	0.00	0.00	0.00	0.00
	D2	4.18	0.70	1.57	0.70	0.87
	NA	5.10	0.17	0.51	0.17	0.34

Values followed by * show significant differences at the 5% level in a Chi square test for homogeneity when compared individually with the other two treatments. Induced callus (IC), number of calluses regenerating green plantlets (CRGP), number of green plantlets regenerated (GPR), number of double haploid plantlets regenerated (DHPR) and number of green double haploids plantlets regenerated (GDHPR)

treatments were similar, but the D2 treatment had slightly higher values for the majority of the parameters.

The percentage of induced calluses (IC) in H28 with the D1 treatment was significantly lower ($P < 0.05$) than the D2 and NA treatments. Therefore, in D1 treatment, parameters that are dependent on IC (CRGP, RGP, RGDHP and RGDHP) were zero, due to a low callus induction. No culture parameters between D2 and NA for H28 were statistically significantly ($P < 0.05$). Although D2 showed higher values than NA for callus that regenerates green plantlets (CRGP), regenerated green plantlets (RGP), regenerated double haploid plantlets (RDHP) and regenerated green double haploid plantlets (RGDHP), these differences were not significant ($P < 0.05$).

Effects of colchicine treatment on callus induction and plantlet regeneration

Callus induction was observed in almost all conditions assayed for both genotypes with the colchicine-supplemented treatments (Table 3). The only exceptions were the 150/24-NA and 300/24-NA media for NRCV980385, with both media used as 24 h colchicine treatments. On one hand, IC in NRCV980385 in the colchicine treatments was significantly lower ($P < 0.05$) than the control. On the other hand, IC for H28 seemed to increase with colchicine, showing significant differences ($P < 0.05$) in both colchicine treatments over 48 h. Moreover, the values were higher in comparison to their respective controls (0/0). Finally, several 24 h colchicine treatments in H28, such as 150/24, 300/24 for D1 and 150/24 for NA, had significantly higher percentages of induced calluses ($P < 0.05$) than their respective controls (0/0-D1 and 0/0-NA).

Regenerated plantlets were obtained from thirteen and fourteen out of fifteen different media for NRCV980385 and H28, respectively. The exceptions were the NA treatments supplemented with colchicine for 24 h (150/24-NA and 300/24-NA) in NRCV980385 and 0/0-D1 in H28. Plantlets regenerated from calluses were either albino or green, although some calluses were capable of regenerating both (Figs. 2, 3 for NRCV980385 and H28 respectively). There was a tendency for higher numbers of albino plantlets to be present when there was a high rate of plantlet regeneration. Albino plantlets regenerating calluses were the most

Table 3 Induced callus (IC) in all treatments assayed for the temperate japonica rice variety, NRVC980385, and the temperate japonica F2 hybrid, H28

	NRCV980385			H28		
	D1	D2	NA	D1	D2	NA
0/0	6.05	7.66	6.96	0.18	4.18	5.10
150/24	7.39	2.40*	0.00*	6.44*	2.73	8.67*
150/48	2.39*	3.51*	7.80	14.10*	17.73*	11.39*
300/24	5.23	3.44*	0.00*	10.55*	3.28	1.37*
300/48	5.81	5.12	8.56	18.75*	9.63*	12.54*

Values followed by * show significant differences at the 5% level in a Chi square test for homogeneity when compared with its hormone control colchicine-free medium (0/0)

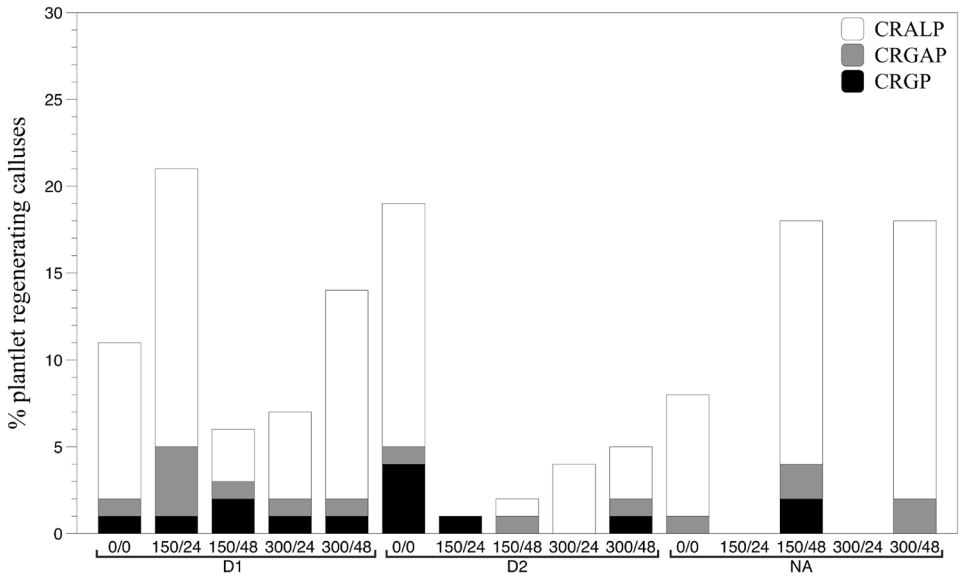

Fig. 2 Percentage of plantlets regenerating calluses for each induction media assayed in NRVC980385. *CRALP* callus regenerating albino plantlets, *CRGP* callus regenerating green plantlets, *CRGAP* callus regenerating green and albino plantlets

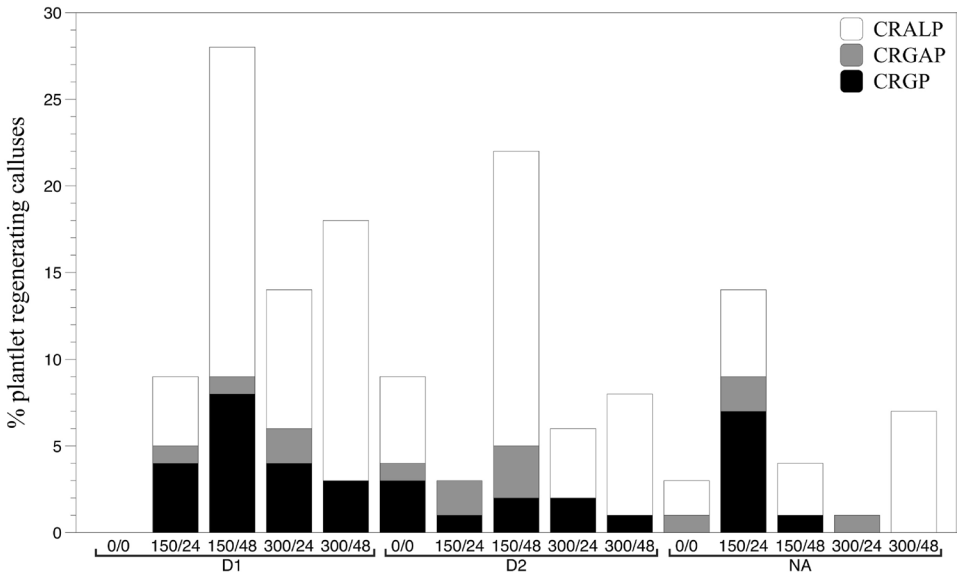

Fig. 3 Percentage of plantlets regenerating calluses for each induction media assayed in H28. *CRALP* callus regenerating albino plantlets, *CRGP* callus regenerating green plantlets, *CRGAP* callus regenerating green and albino plantlets

frequent, representing 77% of and 75% of NRCV980385 and H28 plantlets, respectively, when all media were grouped together.

NRCV980385 regenerated green plantlets in all D1 hormone media (1 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin). In contrast, under D2 hormone conditions (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} kinetin) the values for the total number of calluses regenerating plantlets were higher under colchicine-free conditions, with the number of green plantlets also being higher. Plantlets regenerated in the 150/24-D2 treatment were all green, unlike 300/24-D2 where all plantlets were albino. Finally, 0/0-NA and 300/48-NA conditions caused calluses to regenerate either albino plantlets or albino and green plantlets, but none of the calluses regenerated green plantlets alone.

The ability of H28 calluses to regenerate green plantlets was higher than NRCV980385 in almost all conditions (Fig. 3). Furthermore, colchicine treatments combined with D1 and NA hormone conditions displayed higher CRGP than their control treatments (0/0). Meanwhile, D2 hormone treatment without colchicine regenerated a higher number of green plantlets compared to colchicine treatments with this hormone, a tendency also observed for NRCV980385. Finally, 0/0-NA, 300/24-NA and 300/48-NA conditions had no calluses that regenerated green plantlets exclusively.

Effects of colchicine treatment on RDHP, G/A and RGDHP

The hormone factor has been grouped for both genotypes with the aim of analysing the colchicine effect on regenerated double haploid plantlets (RDHP), the green/albino plantlet ratio (G/A) and regenerated green double haploid plantlets (RGDHP) (Table 4). Values of RDHP and RGDHP for NRCV980385 were greater than for H28, but on average the G/A ratio was lower in NRCV980385 (Table 4). In addition, RDHP values for H28 in the four colchicine treatments at 48 h were higher than in the control, with both treatments showing significant differences.

Table 4 Regenerating double haploid plantlets per 100 anthers plated (RDHP), green/albino ratio (G/A) and regenerating green double haploid plantlets per 100 anthers plated (RGDHP) by genotypes according to colchicine treatment

Colchicine treatment	RDHP		G/A		RGDHP	
	NRCV 980385	H28	NRCV 980385	H28	NRCV 980385	H28
0/0	3.89	0.23	0.19	0.67	0.18	0.00
150/24	2.38	0.54	0.14	1.33	0.49*	0.22
150/48	1.77*	2.26*	0.28	0.55	0.22	0.29
300/24	1.80*	0.46	0.09	0.65	0.31	0.12
300/48	4.22	1.34*	0.12	0.20	0.75*	0.17

Three concentrations were assayed: 0 (control condition), 150 and 300 mg L^{-1}. For each colchicine concentration two exposure times, 24 and 48 h, were tested. In the end, 5 colchicine treatments were tested and named according to concentration/exposure time: 0/0 (control), 150/24, 150/48, 300/24 and 300/48. Hormone factors has been grouped. RDHP and RGDHP values followed by * are significantly different at the 5% level in a Chi square test for homogeneity in comparison with colchicine-free media (0/0)

On the most part, the G/A ratio for NRCV980385 and H28 was not significantly affected by the colchicine treatments. The one exception was H28 in the 150/24 colchicine treatment, which had a doubled G/A ratio in comparison to the colchicine-free control.

Calluses from all colchicine treatments regenerated green double haploid plantlets and the values were similar. The values for regenerated green double haploid plantlets (RGDHP) were always higher on colchicine treatments than on the colchicine-free treatment for both genotypes, with NRCV980385 having the highest values. For NRCV980385, the 150/24 and 300/48 colchicine treatments were significantly different ($P < 0.05$) from their 0/0 controls, with the number of regenerated green double haploid plantlets being 2.5 and 4 times higher respectively. For H28, the RGDHP values showed no significant differences when compared to its control due to the absence of regenerating green double haploid plantlets on 0/0. The best colchicine treatments for H28 were 150 mg L^{-1} at 24 and 48 h.

Haploid diploidization

Application of antimitotic agents to haploid plantlets obtained from the anther culture procedure hindered the rate of plantlet survival. After antimitotic treatment, most analysed plantlets were haploids (Table 5). Plantlets showing only double haploid ploidy were observed at the lower antimitotic concentrations (250 mg L^{-1} of colchicine and 1.25 mg L^{-1} of oryzalin). Within the plantlets that changed his chromosome content, mixiploids, including double haploid ploidy, were the majority. Moreover, when a higher antimitotic concentration was used the plantlet mortality rate increased, reaching 91.11% mortality with 1000 mg L^{-1} of colchicine. In the case of oryzalin, the mid-range concentration treatment (2.5 mg L^{-1}) showed the highest mortality, with a value of 34.78%. The percentage of plantlets that remained haploid after the treatment was higher when the antimitotic oryzalin was used (Table 5) at any concentration. The percentage of haploid plantlets and dead plantlets tended to increase with increases in the antimitotic concentrations.

Discussion

Anther culture is a powerful technique to produce rice DH plants. Nevertheless, the genotype effect is the major limiting factor, causing a differential response in callus induction as well as plantlet regeneration, ploidy and pigmentation previously reported by a number of authors (Mishra and Rao 2016; Herath et al. 2010; Khanna and Raina 1998; Raina and Zapata 1997; Moloney et al. 1989). Additionally, obtaining a high number of regenerating calluses is essential to increase the number of green double haploid plantlets displaying differential genotypes. Regenerated plantlets from the same callus or calluses from the same anther are more likely to be clones and therefore will have poor genetic variability. In addition, due to the fact that anther culture is a two-step process (i.e. initial development of calluses and subsequent regeneration of green plantlets from embryogenic calluses), researchers interested in obtaining new rice varieties from anther culture must avoid bottlenecks in the procedure. Low callus induction, low green plantlet regeneration and low double haploid regeneration can drastically limit the outcomes of anther culture. To minimize this, we proposed a workflow to study the response of the desired genotypes to anther culture over a six-month period using a range of induction media. Consequently, this study has focused on making preliminary assays to determine factors that could improve the yield of green double haploid plants. Additionally, the procedure has reported ways of reducing the amount of work to obtain DH plants in rice by: (i) reducing the time needed for the anther culture procedure, (ii) introducing a fast ploidy determination method, and (iii) a post anther culture diploidization protocol for rice.

Table 5 Post anther culture parameters of treated plantlets

Antimitotic	Concentration (mg L^{-1})	Post anther culture parameters					
		Total plants	% n	% 2n	% 2n mixploid	% mixploid	% dead
Control	0	20	100	0.00	0.00	0.00	0.00
Colchicine	250	47	38.30	2.13	29.79	2.13	27.66
	500	37	18.92	0.00	35.14	0.00	45.95
	1000	45	4.44	0.00	4.44	0.00	91.11
Oryzalin	1.25	31	77.42	6.45	3.23	0.00	12.90
	2.5	46	60.87	0.00	2.17	2.17	34.78
	5	47	70.21	0.00	6.38	0.00	23.40

Percentage n percentage of haploid plantlets with only a level of haploidy, *Percentage 2n* percentage of doubled haploid plantlets that with only a level of diploidy, *Percentage 2n mixiploid* percentage of double haploid plantlets with multiple ploidy levels, including 2n, *Percentage mixiploid* percentage of plantlets with multiple ploidy levels, excluding 2n

We included in our study the NRCV980385 genotype and the growth regulator 2,4-*D* in the induction medium at 2 mg L^{-1} in order to compare results with Serrat et al. (2014). In fact, the number of induced calluses was higher in our study than the previous one, which in turn resulted in a greater number of green double haploid plantlets. The cold pre-treatment was the main difference between the studies, which was changed from 7 to 10 °C and adjusted from a variable 7–12 days to a fixed 9 days. Another factor that could have affected the results is that in this experiment NRVC980385 genotype was a stabilized commercial genotype rather than a heterozygous seed batch as used previously in Serrat et al. (2014). Many authors have confirmed that cold pre-treatment has a stimulatory effect on androgenic response in several genotypes (Tian et al. 2015; Herath et al. 2010; Touraev et al. 2009). Moreover, a temperature of 10 °C is commonly used as a cold pre-treatment (Rukmini et al. 2013; Naik et al. 2017). Indeed, Naik et al. (2017) described that 7 days at 10 °C resulted in the best callus induction in a japonica cultivar. Therefore, the changes in cold pre-treatment enhanced the anther culture protocol with a higher rate of induced calluses.

Among the auxins, 2,4-dichlorophenoxyacetic acid (2,4-*D*) and naphthaleneacetic acid (NAA) are the most commonly used hormones for rice callus induction from anthers (Trejo-Tapia et al. 2002). The colchicine-free D2 treatment (2 mg L^{-1} of 2,4-*D* and 1 mg L^{-1} of kinetin), induced the best culture conditions overall for both the NRVC980385 and H28 genotypes. These results are in agreement with many authors who have determined that 2 mg L^{-1} of 2,4-*D* results in the best culturability results for many genotypes (Chen et al. 2002; Herath et al. 2008). The effect of 2,4-*D* may be the promotion of rapid cell proliferation and formation of non-embryogenic callus as described for spring wheat (Ball et al. 1993). In addition, this auxin at this concentration is widely used in rice anther culture, although regularly combined with other auxins or other cytokines to obtain the best results (Serrat et al. 2014; Afza et al. 2000; Kaushal et al. 2014; Chen et al. 2002). In contrast, the effect of NAA, which is also commonly used in rice anther culture, may be to induce direct androgenesis (Yi et al. 2015; Alemanno and Guiderdoni 1994; Reiffers and Freire 1990). Finally, many authors use combinations of 2,4-*D* and NAA, to obtain better results (He et al. 2006; Xie et al. 1995). Nevertheless, our results do not show clear patterns of rice anther culturability between the two growth regulators.

A differential anther culture response was observed when adding colchicine to the induction medium, which depended on the genotype cultured. NRCV980385 callus induction was negatively affected by colchicine. On the other hand, H28 induced more calluses on colchicine-supplemented induction media. In maize, an increase in embryo frequency has been reported in the presence of colchicine (Barnabás et al. 1999; Obert and Barnabás 2004). In wheat, an absence of effect of colchicine on microspore embryogenesis has been reported by Barnabás et al. (1991), and a reduction was reported by Navarro-Alvarez et al. (1994). In the present study, 75% of the colchicine-supplemented induction media assayed for H28 had significant callus induction enhancement. Moreover, H28 callus induction increased in both colchicine concentrations at 48 h for all of the growth regulators assayed. This effect is also in accordance with Alemanno and Guiderdoni (1994), who described a significant (50%) increase in rice anther callusing with 500 mg L^{-1} of colchicine at 24 and 48 h.

The ability to regenerate plantlets, and specifically green plantlets, was different between the genotypes and the media assayed. NRCV980385 in colchicine-supplemented induction media had no positive effects on green plantlet regeneration. Indeed, treatments with 2,4-*D* at 1 mg L^{-1} regenerated as efficiently with colchicine-supplementation as the colchicine-free control. The ability of H28 to regenerate plantlets from calluses was greater than NRCV980385. At 1 mg L^{-1} of 2,4-*D*, colchicine increased the percentage of regenerating calluses, which was defined as the ability to regenerate higher numbers of green plantlets. Alemanno and Guiderdoni (1994) doubled the number of green plantlet-regenerating calluses with 250 mg L^{-1} of colchicine for 24 h relative to the colchicine-free treatment using the Miara genotype. In contrast, colchicine in the regenerating medium at 30 mg L^{-1} increased 7 times the number of green plantlets in comparison to the control in the Zao jing 26 genotype (Chen et al. 2002). Like these earlier reports, the number of calluses that regenerate green plantlets in H28 genotype, was increased. It is clear from the current work and previous reports that there is a strong effect of genotype on the outcome of anther culture.

In our study, the incidence of albinism was high for both genotypes. Albinism in plants is characterized by a lack of chlorophyll pigments and/or incomplete differentiation of chloroplast membranes in normally green tissues. Many studies have suggested that the use of colchicine in anther culture reduces the albinism ratio (Kumari et al. 2009; Barnabás et al. 1991; Ferrie et al. 2014). In our study, a reduction in albinism was only observed with 150 mg L^{-1} of colchicine during the 24 h treatment of the H28 genotype. This observation is in agreement with other authors who have noted no increases in the proportions of green plantlets after colchicine treatment at the callus stage (Hansen and Andersen 1998; Alemanno and Guiderdoni 1994). Furthermore, the number of double haploid regenerated plantlets also seems to be unaffected by colchicine treatments, irrespective of the different concentrations and exposure times tested. These results are in contrast with reports of an increase in regenerating double haploid plantlets when using colchicine (Barnabás et al. 1991). In our work, the

ploidy of green and albino plantlets was analysed to obtain the parameter of regenerated double haploid plantlets (RDHP), whereas other authors have usually only considered the regenerated green plantlets. Nevertheless, in our study, colchicine-supplemented media increased the proportion of regenerated green double haploid plantlets (RGDHP), which is in accordance with other studies (Alemanno and Guiderdoni 1994; Barnabás et al. 1991; Weber et al. 2005). Finally, all colchicine treatments yielded higher proportions of regenerated green double haploid plantlets for both genotypes in comparison to the colchicine-free induction media. In NRCV980385, the 150 mg L^{-1} colchicine treatment for 24 h and the 300 mg L^{-1} treatment for 48 h gave the best results ($P < 0.05$) compared to 0/0 control, and the numbers of RGDHPs were 2.5 and 4 times greater than the control, respectively. The H28 genotype was not able to regenerate green double haploid plantlets in colchicine-free induction media, and because of that a statistical test was not possible. This lack of green double haploid plantlets from colchicine-free media may be due to a low endoreduplication or low ability for endomitosis in H28, which entails a spontaneous duplication of chromosomes from the haploid (Chen and Chen 1980).

In both genotypes assayed, colchicine seemed to affect the endomitosis rate in the treated microspores. Endomitosis is described as nuclear chromosome doubling due to a failure of the spindle during metaphase (Kasha 2005). C-mitosis is a form of endomitosis caused by colchicine, which has the ability to abort mitosis and inhibit tubulin polymerization in animal and plant cells (Fltzgerald 1976; Pickett-Heaps 1967; Kasha 2005), and this explains the high yield of green double haploid plantlets in rice. Our observed 2.5-fold increase in the proportion of calluses regenerating diploid green plantlets is in accordance with the work of Alemanno and Guiderdoni (1994). Chen et al. (2002) also observed an increase in regenerated green double haploid plantlets when using regeneration media fortified with 75 mg L^{-1} colchicine, although higher concentrations caused harmful effects on calluses and regenerated plantlets. For this reason we assayed 300 and 150 mg L^{-1} concentrations to test lower concentrations than the 500 mg L^{-1} used by Alemanno and Guiderdoni (1994) and delimit the best colchicine concentration while avoiding toxicity. Finally, colchicine is widely used to increase the number of green double haploid plantlets in anther culture of other species and has had positive results in wheat (Hansen and Andersen 1998; Soriano et al. 2007), maize (Saisingtong et al. 1996), oats (Ferrie et al. 2014) and rapeseed (Mollers et al. 1994; Weber et al. 2005). Despite our results and those reported in the literature, we suggest that further studies should be performed in rice to investigate the ability of colchicine to increase the numbers of regenerated green double haploids above albino double haploids.

The main limitation of anther culture is the unknown interaction that occurs between media and genotypes. Our results describe completely different responses for callus induction, regenerating calluses, plant albinism and the ploidy of regenerated plantlets that are dependent on the growth regulator, their concentrations and the exposure time. Many authors have reported that the genotype affects the androgenic response (Lentini et al. 1997; He et al. 2006; Bagheri and Jelodar 2008) and that changes in medium composition can alter the response of different rice cultivars (Trejo-Tapia et al. 2002; Chen et al. 2002; Herath et al. 2008, 2010). However, manipulation of colchicine in induction media has not been reported previously for rice.

A complementary way to obtain DH plants is to perform a post anther procedure treatment of green haploid plantlets with antimitotics. It has been widely reported that antimitotic treatments of plantlets may change their ploidy (Chen et al. 2002; Ascough et al. 2008; Gallone et al. 2014; Sarathum et al. 2010; Omidbaigi et al. 2012; de Carvalho et al. 2005). In this study, the in vitro production of double haploid plantlets from already formed haploid plantlets was achieved. Most of the plantlets that survived were mixiploid with levels of diploidy, and it was from these latter plants that we were able to obtain double haploid seed from those tillers that were double haploids. Colchicine at 500 mg L^{-1} was the best in vitro treatment to double the ploidy (35.14% of plantlets treated). This concentration of colchicine was also used previously in an in vivo treatment of tillers with an effectiveness of 11.5% (Chen et al. 2002). Ascough et al. (2008) reported that when lower antimitotic concentrations were used the number of surviving plantlets was higher, but on the other hand the level of diploidization was also lower. Omidbaigi et al. 2012 reported that high concentrations of oryzalin did not have much effect on the survival ratio.

Anther culture in rice has been studied in many genotypes to achieve the best method of maximizing green double haploid plantlet formation through different stresses. Taking this earlier work into account, we selected a range of stresses to formulate protocols for two Mediterranean japonica rice genotypes that will form the basis of an anther culture procedure for a wide range of genotypes. The genotypes trialled each had specific responses to the experimental conditions. We have demonstrated that cold pre-treatment at 10 °C for 9 days increases callus induction. Without colchicine in the induction medium, we recommend 2,4-*D* at 2 mg L^{-1} and kinetin at 1 mg L^{-1} to obtain the highest values for callus induction and green double haploid plantlet regeneration for Mediterranean japonica rice varieties. Colchicine-supplemented induction media may increase the level of callus induction, depending on the genotype. Colchicine on the induction medium increases the green double haploid plantlet production in all treatments of concentration and time assayed. We have shown that post-anther culture colchicine

treatment at 500 mg L^{-1} increases the green double haploid production from green haploid plantlets. Our results highlight the importance of the genotype and media interactions effects on the anther culture efficiency in rice. This study stands out the necessity to continue studying the response of rice to anther culture to better understand the main mechanism of interaction between genotype and induction media.

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