Rev. Biol. Trop., 44(3)/45(1): 13-21, 1996-1997

Plant regeneration of *indica* rice (*Oryza sativa*) cultivars from mature embryo-derived calli

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(Rec. 28-IX-1995. Rev. 5-XII-1995. Acep. 31-I-1996)

Abstract: Plant regeneration from seven-week-old callus cultures derived from mature embryos of several *indica* rice cultivars was achieved with frequencies of morphogenic calli from 10 to 47 %. Three media were tested both for callogenesis and plant regeneration. For 3 of the 7 genotypes examined, the best combination of media for plant regeneration was Murashige & Skoog basal medium: MSC (callogenesis) and MSR (regeneration). The rates of callogenesis were not related to the capacity for plant regeneration. Two genotypes CR-1113 and CR-5272 produced the highest number of regenerated green plants. The results of this study suggest that genetic differences could be directly linked to the ability to regenerate in these plant cultivars.

Key words: *Oryza sativa*, *indica* rice, tissue culture, plant regeneration.

Rice is the most important staple crop for one-third of the world's population, and the *indica* cultivars are mostly grown in developing countries. In Costa Rica, these cultivars provide about 40 % of the population's daily caloric intake. Preliminary surveys indicate that all commercial rice genotypes are susceptible to rice hoja blanca virus (RHBV), a member of the tenuivirus group, and that 40 to 80 % of plants are infected with RHBV, reducing the biomass by up to 50 % (Rodríguez *et al.*, non published results). Resistance to this disease has been found only in *japonica* cultivars (Zeigler *et al.* 1988, Tohme *et al.* 1991); it cannot be transferred to *indica* varieties by conventional breeding without loss of agronomic and culinary characteristics. Therefore, selective transfer of non-conventional disease resistance to the rice genome by plant transformation using viral genes is a priority. For this transformation to be successful, plant regeneration from highly embryogenic somatic cultures or cell suspensions must be further developed (Vasil 1990).

The regeneration of rice plants has been obtained in tissue cultures from meristematic organs such as root segments (Kawata & Ishihara 1968, Abe & Futsuhara 1984 1985, Kavi Kishor & Reddy 1986); leaf bases of young seedlings (Wernicke *et al.* 1981); mature embryos (Inoue & Maeda 1980, Abe & Futsuhara 1984 1986, Raghavan Ram & Nabors 1984, Boissot 1990, Chowdhry *et al.* 1993); immature embryos (Heyser *et al.* 1983, Raina *et al.* 1987, Hartke & Lorz 1989, Koetje *et al.* 1989, Boissot 1990); and young inflorescences (Chen *et al.* 1985). Competence for callus induction and plant regeneration in rice is genetically controlled, as was first demonstrated by Abe & Futsuhara (1986), who studied more than 500 varieties. *Japonica* rice varieties display higher rates of callus induction and plant regeneration than *indica* genotypes. Although for most *indica* genotypes the establishment of embryogenic cell cultures is very difficult, plant regeneration has been obtained from mature and inmature embryos and from anthers of several *indica* genotypes (Raina *et*

al. 1987, Koetje *et al.* 1989, Boissot 1990, Gosal *et al.* 1991).

The objective of this research was to develop experimental conditions for plant regeneration from somatic tissue cultures of commercial Costa Rican *indica* rice cultivars so that genetic engineering can be used to confer resistence to RHBV.

MATERIALS AND METHODS

Plant material and embryo isolation: Seeds from five commercial cultivars (CR-201, CR-1113, CR-5272, CR-1707, and CR-1821) and from two experimental lines (CR-8334, and CR-8341) were supplied by the National Rice Office, Costa Rica. Seeds were surface-sterilized in an aqueous 30% (vol/vol) Domestos™ (Lever Ltd. Warrington, G.B.) solution for 35 min, and then rinsed five times in sterile distilled water. Disinfected seeds were soaked overnight in sterile distilled water to facilitate embryo isolation without damaging the scutellum. Embryos were excised under a binocular microscope using fine scalpels and forceps.

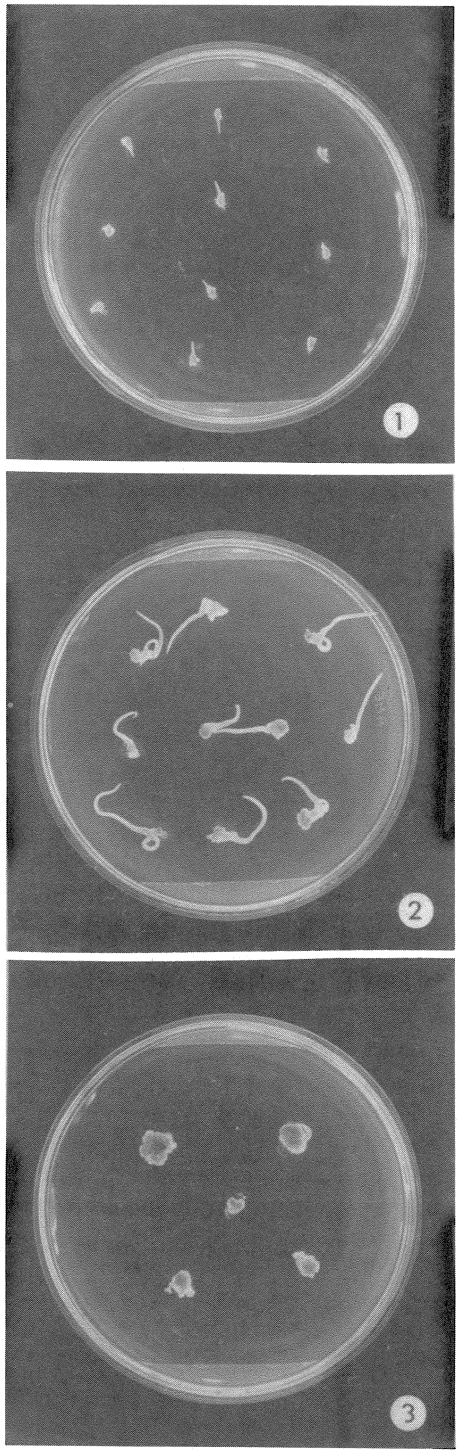
Callus induction and plant regeneration: Ten aseptic mature embryos were plated per petri dish (100 x 15 mm) containing 40 ml of culture medium for callus induction, with the scutellum in contact with the medium. The number of explants used varied between 90 and 220 for each experiment. Three media were examined for callus induction: two MS basal media (Murashige & Skoog 1962), one of them supplemented with 2.5 mgL^{-1} 2,4-D, and the other with 2.0 mgL^{-1} 2,4-D and 50 mgL^{-1} tryptophan, and one N_6 basal medium (Chu *et al.* 1975) supplemented with 1 gL^{-1} hydrolyzed casein and 0.5 mgL^{-1} 2,4-D. These three basal media have been successful for callus induction and subsequent plant regeneration in several *indica* genotypes as reported by several authors (Hartke & Lörz 1989, Koetje *et al.* 1989). All basal salt media were supplemented with 10^{-4} M FeEDTA, 1 mgL^{-1} thiamine, 100 mgL^{-1} *myo*-inositol, 3% (w/v) sucrose, and solidified with 0.3 % (w/v) Phytagel™. The pH of all media was adjusted to 5.8 before autoclaving at 120 °C for 20 min. Petri dishes containing isolated embryos were sealed with plastic (Glad-Cling Wrap) and incubated at 25 °C in the dark. After 3 weeks of embryo culture, calli from the scutellum were isolated and subcultured in the same medium for a further period of 4 weeks. Calli were then transferred onto the same medium (MS or N_6) without growth regulators, or to regeneration medium MSR (MS basal medium supplemented with 0.5 mgL^{-1} BAP and 0.05 mgL^{-1} NAA), and were subcultured monthly for three months in order to assess their morphogenic ability. For plant regeneration, cultures were maintained under a 16-h photoperiod provided by cool-white fluorescent lamps (2000 lux). Plantlets of 10 to 15 cm height were transferred to sterile-soil pots and protected with plastic bags for acclimatization before being moved to the greenhouse for further growth and maturation.

Statistical analysis: The numbers of calli were analysed to obtain means and standard errors for two-way analysis of variance for unbalanced data, and for Fisher's LSD (least significant difference) using the statistical procedure STATVIEW 4.0 for Macintosh.

Abbreviations: 2,4-D: 2,4-dichlorophenoxyacetic acid;
BAP: 6-benzylaminopurine; NAA: naphthalene acetic acid

RESULTS

Mature embryos (Fig. 1) produced nodular and yellowish calli, derived from the scutellum (Fig. 2) after two weeks of culture. They also formed hydric calluses which were not able to regenerate plants when plated on differentiation medium. Callus induction (Table 1) varied from 0.71 calli per ten explants (7.1 %) for CR-8334 on MSTf medium to 9.0 (90 %) for CR-1707 on MSC medium. The two-way analysis of variance for means of number of calli produced (Table 2) shows that the effect of the genotype and of the media was highly significant ($p<0.0001$ for both). A highly significant interaction ($p<0.0001$) between genotypes and treatments was also found by this analysis. This suggests that the genotypes react differently according to the basal salt medium for callogenesis. Table 3 shows the Fisher's least significant difference for means of number of calli



Figs. 1-3. Calli originated from embryo cultures of the Costa Rican rice cultivar CR-5272. 1- Mature embryos on MSC medium; 2- Two-week-old calli; 3- Four-Week-old calli.

produced per ten explants plated. The greatest differences were found between genotypes CR-8341 and CR-8334, and the other genotypes. These two genotypes display lower rates for callogenesis (Table 1) in all the treatments. This analysis suggests that there are two groups of genotypes in terms of their ability for callogenesis: CR-8341 and CR-8334 with low rates, and CR-201, CR-1707, CR-1821, CR-1113, and CR-5272, with higher rates of callogenesis in all the treatments.

Calli transferred onto regeneration medium grew considerably during the first four weeks of culture (Fig. 3). The ability of these calli to regenerate shoots and plantlets after twelve weeks of culture on regeneration medium is presented in Table 4 and Fig. 4. For three (CR-201, CR-1113 and CR-5272) of the seven genotypes examined, the best experimental conditions were obtained with MSC and MSR media for callogenesis and plant regeneration, respectively. Nevertheless, according to results presented on Table 1, the rates of callogenesis were not related to the morphogenic ability of the calli: the genotypes displaying the highest frequencies for callus induction in all media tested regenerated none or only a few plantlets (Table 4). The genotype CR-201 produced 12.5 % of morphogenic calli, with a mean number of 2.3 shoots per callus plated. A total of 8 plants were regenerated from this genotype. The genotype CR-1113 generated 22.7 % of morphogenic calli, with an average of 1.2 shoots per callus plated and 12 regenerated plants after the treatment MSC/MSR. The genotype CR-5272 displays the highest rate of morphogenic calli (46.9 %), with a mean of 3.5 shoots per callus examined; a total of 51 plants (Fig. 5) were obtained. The remaining genotypes did not produce morphogenic calli. The regenerated plants are being grown in the greenhouse until maturation and will be evaluated for fertility and morphological stability. No albinos were recovered for all the genotypes examined.

DISCUSSION

Several authors studied the genetic composition of the rice cultivars planted during 1973-1989 in Latin America and the Caribbean, and found that nearly 56 % of the

TABLE 1

Means and standard errors of number of calli per ten explants plated, derived from mature embryo cultures

Cultivars	Culture media					
	MSC		MSTf		N_6 Cs	
	Mean	SE	Mean	SE	Mean	SE
CR-201	6.82 n=170	0.50	7.00 n=140	0.47	7.55 n=90	0.73
CR-1113	5.55 n=200	0.34	6.35 n=200	0.45	6.15 n=190	0.55
CR-1707	9.00 n=200	0.25	4.90 n=200	0.41	6.60 n=200	0.34
CR-1821	8.22 n=220	0.35	5.50 n=180	0.50	9.15 n=190	0.25
CR-5272	6.00 n=160	0.43	7.14 n=140	0.50	7.07 n=140	0.80
CR-8334	1.18 n=160	0.26	0.71 n=140	0.25	2.50 n=180	0.36
CR-8341	0.73 n=190	0.20	1.00 n=200	0.3	1.75 n=200	0.32

SE: Standard error
MSC: Murashige & Skoog (1962) + 2,5 mgL^{-1} 2,4-D
MSTf: Basal MS + 2,0 mgL^{-1} 2,4-D + 50 mgL^{-1} Triptophan
N_6Cs: Basal N_6 (Chu *et al.* 1975) + 0,5 mgL^{-1} 2,4-D + 1 g L^{-1} hydrolised casein

TABLE 2

Two-way analysis of variance (ANOVA) for means of number of calli from mature embryo cultures of seven rice cultivars cultured on three basal media

Source of variation	DF	Sum of squares	Mean square	F-value	Probability
Genotype	6	2409.838	401.640	134.903	< .0001
Medium	2	79.092	39.546	13.283	< .0001
Genotype + medium	12	273.050	22.754	7.643	< .0001
Residual	348	10366.081	2.977		

TABLE 3

Fisher's LSD (least significant difference) for means of number of calli from mature embryo cultures

Genotypes	Mean	Difference	Probability
CR-201,	CR-1113	1.033	0.0037
CR-201,	CR-170	0.217	0.5388
CR-201,	CR-1821	-0.645	0.0689
CR-201,	CR-5272	0.345	0.3601
CR-201,	CR-8341	5.881	< 0.0001
CR-201,	CR-8334	5.508	< 0.0001
CR-1113,	CR-1707	-0.816	0.0103
CR-1113,	CR-1821	-1.678	< 0.0001
CR-1113,	CR-5272	-0.688	0.0462
CR-1113,	CR-8341	4.847	< 0.0001
CR-1113,	CR-8334	4.475	< 0.0001
CR-1707,	CR-1821	-0.862	0.0068
CR-1707,	CR-5272	0.129	0.7071
CR-1707,	CR-8341	5.664	< 0.0001
CR-1707,	CR-8334	5.292	< 0.0001
CR-1821,	CR-5272	0.990	0.0042
CR-1821,	CR-8341	6.525	< 0.0001
CR-1821,	CR-8334	6.153	< 0.0001
CR-5272,	CR-8341	5.535	< 0.0001
CR-5272,	CR-8334	5.163	< 0.0001
CR-8341,	CR-8334	-0.372	0.2679

genes under commercial exploitation came from the three genotypes that generated the variety IR8, one of the progenitors of the main rice cultivars in the region. Cultivated rice varieties in Costa Rica have a coefficient of parentage (r) of 0.41 because they were developed from only three different genotypes, IR8, Pan Kharl 203, and Colombia 1 (Fig. 6). Such limited variability might generate phytosanitary problems if new virulent strains of the pathogens or more efficient vectors arise. This is particularly relevant to RHBV infection because the progenitor IR8 is susceptible to RHBV (Cuevas-Pérez *et al.* 1992, De Nivia *et al.* unpublished results).

The common ability of cultivars CR-1113 and CR-5272 to regenerate plants under the experimental conditions used in this study suggests that this ability is genetically controlled, and may reflect their high degree of relatedness; they have the same progenitors (Fig. 6). The three genotypes CR-201, CR-1707, and CR-1821 are closely related to each other, but although they share some progenitors with CR-1113 and CR-5272, they do not yield regenerated plants. CR-8341 and CR-8334 were released in 1992 and have different origins. CR-8334 has as progenitors Cica 7 and the product of the

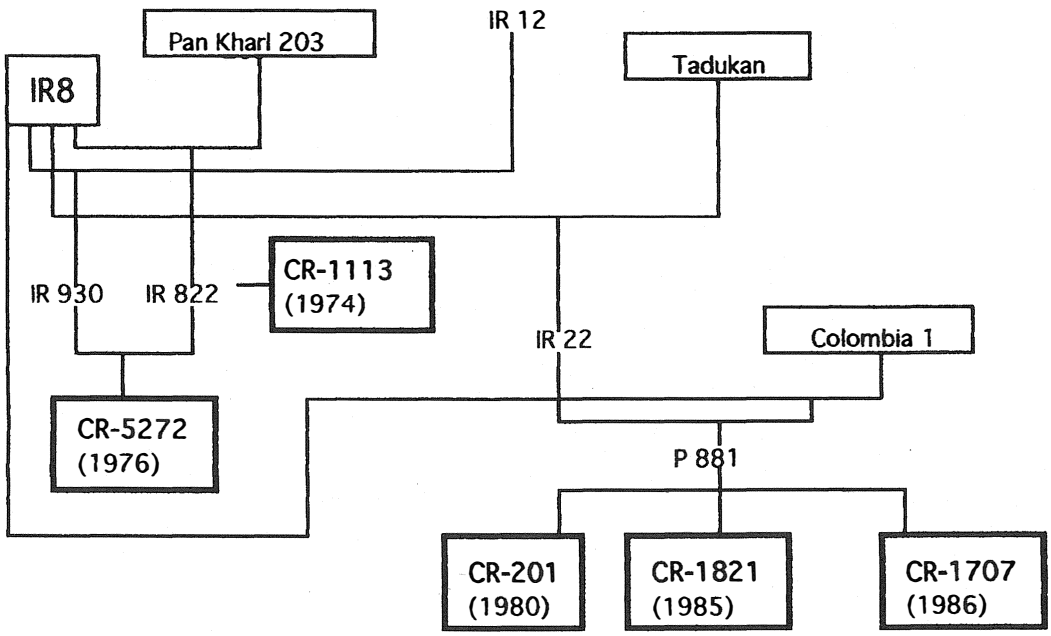


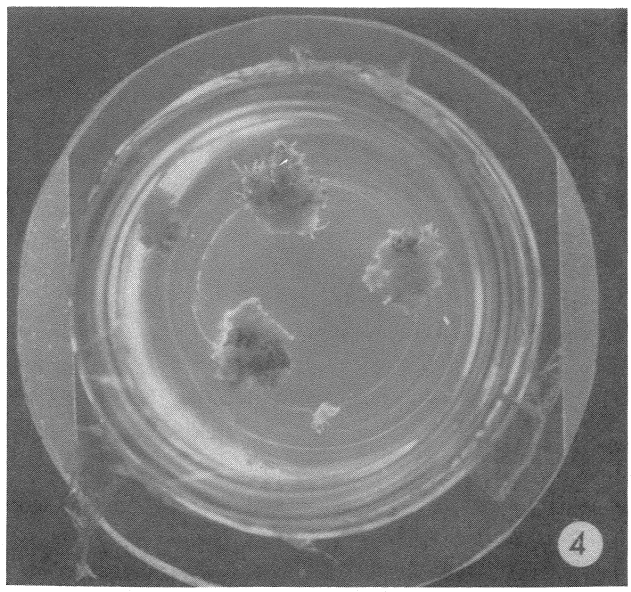
Fig. 6. Phylogeny of the main Costa Rican rice cultivar (according to Cuevas-Pérez *et al.* 1992).

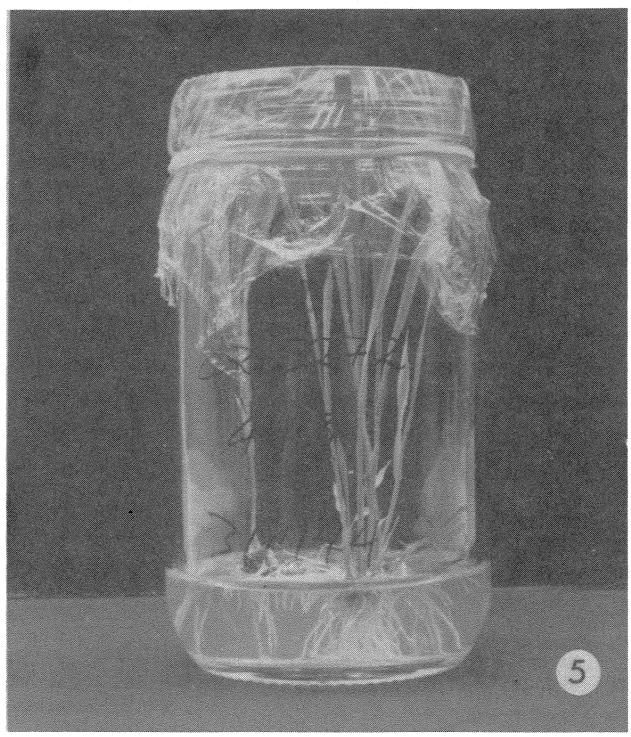
TABLE 4

Effect of callus induction and regeneration media on plant regeneration from mature embryo cultures of seven rice cultivars after twelve weeks of culture

cultivar	Culture media (callogenesis/regeneration)	N° of calli plated	N° of calli with shoots	% morphogenic calli+	Mean N° of shoots by morphogenic calli	N° total of regenerated plants
CR-201	MSC/MSR	48	6	12.5	2.3	8
	MSTf/MSR	21	0	0	0	0
	MSTf/MSTfR	27	0	0	0	0
	N6Cs/MSR	18	0	0	0	0
	N6Cs/N6CsR	17	0	0	0	0
CR-1113	MSC/MSR	44	10	22.7	1.2	12
	MSTf/MSR	48	5	10.4	1.6	8
	MSTf/MSTfR	56	1	1.8	1	0
	N6Cs/MSR	30	0	0	0	0
	N6Cs/N6CsR	30	0	0	0	0
CR-1707	MSC/MSR	52	0	0	0	0
	MSTf/MSR	30	0	0	0	0
	MSTf/MSTfR	18	0	0	0	0
	N6Cs/MSR	45	0	0	0	0
	N6Cs/N6CsR	34	0	0	0	0
CR-1821	MSC/MSR	81	0	0	0	0
	MSTf/MSR	32	0	0	0	0
	MSTf/MSTfR	43	0	0	0	0
	N6Cs/MSR	27	0	0	0	0
	N6Cs/N6CsR	40	0	0	0	0
CR-5272	MSC/MSR	49	23	46.9	3.5	51
	MSTf/MSR	31	3	9.6	1.1	0
	MSTf/MSTfR	38	0	0	0	0
	N6Cs/MSR	16	0	0	0	0
	N6Cs/N6CsR	17	0	0	0	0
CR-8334	MSC/MSR	8	0	0	0	0
	MSTf/MSR	6	0	0	0	0
	MSTf/MSTfR	-	-	-	-	-
	N6Cs/MSR	14	0	0	0	0
	N6Cs/N6CsR	-	-	-	-	-
CR-8341	MSC/MSR	10	0	0	0	0
	MSTf/MSR	6	0	0	0	0
	MSTf/MSTfR	-	-	-	-	-
	N6Cs/MSR	17	0	0	0	
	N6Cs/N6CsR	-	-	-	-	-

+: N° calli with shoots/ Total N° of calli x 100
MSR : MS (1962) + 0.5 mgL^{-1} BAP + 0.05 mgL^{-1} NAA ;
MSTfR: Basal MS + 0.5 mgL^{-1} BAP + 0.05 mgL^{-1} NAA+ 50 mgL^{-1} Triptophane
N_6Cs R: Basal N_6 (Chu *et al.*. 1975) +0.5 mgL^{-1} BAP+0.05 mgL^{-1} NAA + 1 g L^{-1} hydrolized casein





Figs. 4-5. Development of plantlets from mature embryo derived calli of the Costa Rican rice cultivar CR-5272. 4- Shoot development after four weeks on MSR medium; 5- Regenerated plants growing on MSR medium.

cross between 4440 and Pelita 1. CR-8341 is derived from Cica 7 and the hybrid of 5461 and Cica 4 (Tinoco, pers. comm.).

The results of this study suggest that genetic differences are involved in the ability of the studied cultivars to originate somatic tissue cultures and to regenerate plants. Research will be focused on the establishment of embryogenic cell suspension cultures and on the genetic transformation of the morphogenic genotypes, CR-5272 and CR-1113, with viral genes in order to confer resistance to the RHBV disease.

ACKNOWLEDGEMENTS

The authors wish to thank Marta Oliva, Ingrid Rodríguez and Tania Quesada for assistance in tissue culture, Reynaldo Pereira for photographic work, Lawrence Kirkendall for advice and help in statistical analysis, and Julie Simpson, Jean-Vincent Escalant and Joachim de Miranda for revision of the manuscript. This work was supported by the University of Costa Rica (Project N° 111-92-570) and by the Rockefeller Foundation.

RESUMEN

Se obtuvo la regeneración de plantas a partir de cultivos de callos de siete semanas derivados de embriones maduros en varios cultivares costarricenses de arroz *(Oryza sativa)*. La frecuencia de callos morfogénicos estuvo comprendida entre 10 y 47 %. Se examinaron tres medios de cultivo tanto para el proceso de callogénesis como de regeneración. Para tres de los siete genotipos la mejor combinación de medios para la regeneración de plantas fue la de Murashige & Skoog: MSC para callogénesis y MSR para regeneración. Los cultivares CR-1113 y CR-5272 produjeron el mayor número de plantas regeneradas luego de doce semanas de cultivo. Estas plantas se transfirieron al invernadero y serán evaluadas próximamente en términos de fertilidad y de integridad morfológica. Los resultados de este estudio sugieren que diferencias genéticas pueden estar relacionadas con la capacidad para la regeneración de plantas en los genotipos estudiados.

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