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Genetic stability in rice micropropagation

R. Medina, M. Faloci, M.A. Marassi, and L.A. Mroginski.

Instituto de Botánica del Nordeste (IBONE), Facultad de Ciencias Agrarias (UNNE). C.C. 209. Corrientes (3400). Argentina.

Key words: micropropagation, multiple shoots, *Oryza sativa* L., isozyme, genetic stability.

ABSTRACT: An efficient clonal propagation procedure for six rice varieties cultivated in Argentina was developed by using shoot tip cultures, and the genetic stability of the micropropagated plants was verified by isozyme analysis. One week old seedlings obtained on MS medium were sectioned and subcultured on MS medium (0.75% agar) supplemented with different combination and concentrations of cytokinins (BAP and KIN) and auxins (2,4-D and NAA). After four weeks of culture, multiple shoots were obtained. The best response was observed on MS supplemented with BAP 5 mg l^{-1}. Shoot clumps were multiplied in MS liquid medium containing BAP 5 mg l^{-1}. Profuse rooting was obtained after transfer to MS medium lacking growth regulators and with sucrose 8% (w/v). Complete plants were successfully transferred to soil and grown to maturity. ADH and EST patterns of micropropagated rice plants showed polymorphisms compared with plants of the original varieties. However, the zymograms of the seed derived progeny of the micropropagated plants were similar to that of the original varieties. These results indicate the maintenance of the genetic stability in the sexual progeny of micropropagated plants.

Abbreviations: BAP, 6-benzylaminopurine; 2,4-D, 2,4-dichlorophenoxyacetic acid; KIN, kinetin; NAA, 1-naphthalenacetic acid; ADH, alcohol dehydrogenase; CAT, catalase; DIA, diaphorase; EST, esterase; PGI, phosphoglucoisomerase.

Introduction

Oryza sativa L. is the most economically important cultivated rice species of the *Oryzeae* tribe (*Poaceae*). The major ecogeographic *O. sativa* subspecies are *japonica* and *indica*, which can be distinguished by their morphological and physiological characteristics (Chang *et al*., 1991). This self-pollinated species (2n = 2x = 24) is conventionally propagated by seeds. Rice is the world's single most important food crop, and it represents a primary food for more than a third of its population (David, 1991).

Tissue culture techniques have become necessary for mass-production of transgenic rice plants (Peng *et al.*, 1992), hybrids between wild rice and *O. sativa* which have a low seed production (Mariam *et al*., 1996), and for the recovery of germplasm when seed availability is limited. A large number of culture protocols can be used for mass-production of rice. The methods based on adventitious shoot cullture (Khanna and Raina, 1998) and somatic embryogenesis (Rueb *et al*., 1994) may result in genetic variation of the obtained plants which are usually regenerated from callus. On the other hand, shoot primordia and multiple shoot mediated regeneration have been considered to ensure genetic stability (Grout, 1990; Yoshida, 1996). Micropropagation techniques have been established for *japonica* and *indica* rice using as explants either mature seeds (Sandhu *et al*., 1995; Yoshida and Kato, 1996), shoot segments (Pádua *et al*., 1998) or embryos (Yoshida, 1996; Yoshida and Kato,

Address correspondence to: Ing. Agr. Ricardo Medina. Instituto de Botánica del Nordeste, Facultad de Ciencias Agrarias (UNNE). Casilla de Correo 209, (3400) Corrientes, ARGENTINA.
Fax: (+54-3783) 427131; E-mail: ricardomedina@agr.unne.edu.ar
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1996), which produced shoot primordia or multiple shoots.

If mass-production is the aim, the importance of maintaining the stability in regenerated plants should not be ignored. In this sense, genetic stability was evaluated by morphological characterization of rice plants derived from multiple shoots (Yoshida and Kato, 1996) and regenerated plants by somatic embryogenesis (Rueb *et al*., 1994). With the same criterion, seed progeny of plants regenerated by somatic embryogenesis from protoplasts were compared with control seedlings (Abdullah *et al*., 1989), but no biochemical characterization of the derived progeny has been reported.

In this report we compared the effect of six *in vitro* micropropagation media for six rice varieties cultivated in Argentina and verified by isozyme analysis the genetic stability of the sexual progeny derived from the micropropagated plants after four *in vitro* multiplication cycles.

Materials and Methods

Plant material and culture conditions for micropropagation

For donor plantlets production, seeds of rice (*Oryza sativa* L.) subspecies *japonica* (var. 'Mocoi F.C.A.', 'Itapé P.A.', 'Fortuna INTA') and *indica* (var. 'EMBRAPA-7-Taim', 'CT 6919', 'BR IRGA 409'), were dehusked, surface sterilized with ethanol 70% for 3 min, followed by immersion in NaClO 50% (v/v) for 45 min and washed three times with sterile distilled water. Thereafter, the sterilized seeds were cultured on MS (Murashige and Skoog, 1962) medium for germination. After 7 d, the seedlings were sectioned below the first node and the upper part of the coleoptile, and an explant 2 cm long was obtained.

Explants were cultured on MS medium supplemented either with 5 mg l^{-1} BAP (Sandhu *et al.*, 1995) or with 0.2 mg l^{-1} 2,4-D, or 1.8 mg l^{-1} NAA plus 2.15 mg l^{-1} KIN, or 22.5 mg l^{-1} BAP (Yoshida, 1996). The pH of the medium was adjusted to 5.8 using either HCl or KOH prior the addition of 0.75% Sigma agar (A-1296) and autoclaved at 1.46 kg cm^{-2} for 20 min.

The experiment consisted of 30 treatments. Each treatment (variety x growth regulators) was independently replicated three times. Each replication consisted of four glass flasks containing three explants each. Treatments were randomly arranged on the growth room shelves.

The glass flasks (110 cm^3) were covered with Resinite AF-50® film (Casco S. A. C. Company, Buenos Aires) and incubated in a growth room at 27±2°C under a 14 h photoperiod regime with an irradiance of 116 µmol m^{-2} s^{-1} provided by cool white fluorescent lamps.

The percentage of explants forming multiple shoots (multiplication frequency) and the number of shoots derived from each explant (regeneration intensity) were evaluated after 30 d of culture.

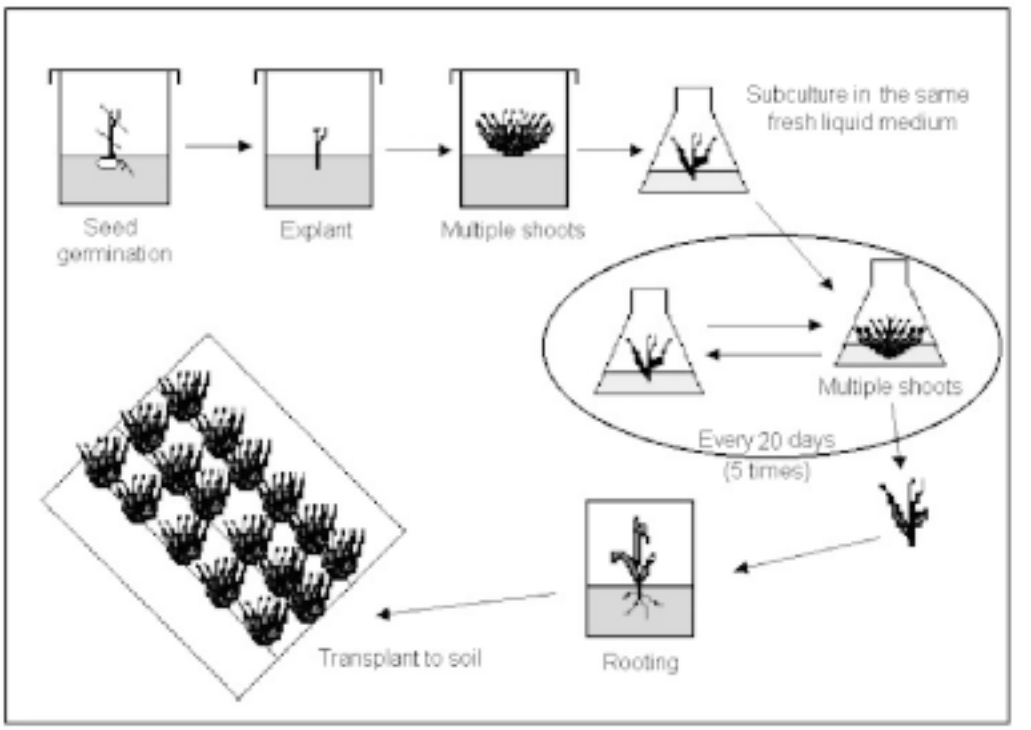

FIGURE 1. Schematic protocol used in this work for rice micropropagation.

Half of the total multiple shoots (4-10 shoots/clump) obtained in varieties 'Mocoi F.C.A.' and 'Itapé P.A.' was subcultured in Erlenmeyer flasks with liquid fresh medium with the same composition, while the rest was transferred to MS medium with sucrose 8% (w/v) for rooting. The subcultures were repeated five times at 20 d intervals.

A schematic representation of the micropropagation protocol followed in the present study can be observed in Figure 1.

The regenerated plants (20 cm long) were removed from the rooting medium; roots were washed and leaves were cut to half their length. The plants were then transferred into glass flasks with tap water and maintained for a week under laboratory conditions. When the plants showed leaf elongation, they were transferred to soil in a greenhouse until they produced seeds that were harvested to evaluate the progeny.

Statistical analysis

Means and standard error (± SEM) were calculated. The data were subjected to analysis of variance (ANOVA) and comparisons of means were made by Duncan's multiple comparison test ($P \leq 0.05$).

Isozyme analysis

Isozyme systems were assayed on samples of the seed derived plants of the original varieties 'Mocoi F.C.A.' and 'Itapé P.A.' used as controls, the micropropagated plants after four multiplication cycles, and their seed derived progeny. Seventy five plants from each type of genetic material were evaluated.

Protein extracts were prepared from young leaves (50% expanded leaf) of control plants, plants derived from the seed of the micropropagated plants and

FIGURE 2. Micropropagation of rice through proliferation of axillary shoots.
a. left: explant cultured on MS medium lacking growth regulators; right: shoot proliferation on MS medium supplemented with 5 mg l^{-1} BAP after 30 d of the incubation. Scale bar: 7 mm.
b. shoot proliferation and formation of clumps during 20 d cycles. Scale bar: 10 mm.
c. induction of rooting *in vitro*. Scale bar: 14 mm.
d. propagated rice plants at maturity. Scale bar: 21 mm.

micropropagated plants transferred to soil. Homogenates were obtained by mechanically grinding with buffer Tris-HCl 0.1 M pH = 6.8 + glycerol 2% + β-mercaptoethanol 0.1% + bromophenol blue 0.01%, in a relation 0.1 g ml^{-1}. Protein extracts were centrifuged at 14,000 rpm for 10 min. The supernatants were used immediately for isozyme analyses or stored frozen at -70ºC.

The isozyme systems assayed were: alcohol dehydrogenase (ADH) catalase (CAT), diaphorase (DIA), esterase (EST) and phosphoglucoisomerase (PGI) on discontinuous native polyacrylamide gels (PAGE) 3% - 7.5% mini slab according to Laemmli (1970). The electrode buffer contained Tris-HCl 0.025 M and glycine 0.192 M, pH = 8.3. Depending on the isozyme system, 10 - 15 µl of the sample was loaded. Electrophoresis was performed at 4ºC during 3 h at constant current, 1.2 mA cm^{-1}. The gels were stained according to Arulsekar and Parfitt (1986) for ADH and PGI; according to Stuber *et al.* (1988) for CAT and DIA, and according to Soltis *et al.* (1983) for EST. The relative electhrophoretic mobility (R_f) was calculated for each isozyme band.

Results

Establishment of shoot cultures and primary multiplication

After 15 d, survival of the cultured explants was evaluated; 50 to 100% of the explants from all culture media and varieties were able to survive. In Table 1, the percentage of explants forming multiple shoots on the different multiplication media is summarized. In varieties 'Fortuna INTA' and 'CT 6919', significant differences were observed in multiplication frequencies when the MS was supplemented with 5 mg/l^{-1}BAP, according to Duncan's multiple comparison test ($P \leq 0.05$). In 'BR IRGA 409', 'Itapé P.A.', 'EMBRAPA-7-Taim' and 'Mocoi F.C.A.', the MS + 5 mg/l^{-1} BAP medium also showed high multiplication frequencies although it was not the only medium with a good behavior. We also found that MS + 5 mg/l^{-1} BAP induced the highest number of shoots derived from each explant in 'Fortuna INTA' and 'CT 6919' (Table 2). For the other varieties

TABLE 1.

Effects of six micropropagation media on the percentage of explants forming multiple shoots in six varieties of rice, after 30 d of culture.

Culture media*	Varieties					
	IRGA 409	ITAPÉ	FORTUNA	TAIM	CT6919	MOCOI
MS	0±0.0 [a]	0±0.0 [a]	0±0.0 [a]	0±0.0 [a]	0±0.0 [a]	44±10.2 [ab]
MS + BAP 5	76±15.4 [b]	62±7.7 [b]	59±25.0 [b]	83±3.3 [d]	84±16.8 [d]	77±14.9 [b]
MS + 2.4-D 0.2 + KIN 2.15	6±9.6 [a]	7±6.7 [a]	7±11.5 [a]	8±8.4 [ab]	7±11.5 [ab]	21±14.5 [a]
MS + 2.4-D 0.2 + BAP 22.5	57±23.3 [b]	8±8.4 [a]	11±19.2 [a]	40±26.7 [bc]	31±30.1 [bc]	57±9.2 [ab]
MS + NAA 1.8 + KIN 2.15	52±13.5 [b]	54±25.9 [b]	12±5.1 [a]	71±30.1 [cd]	50±3.3 [c]	61±22.9 [b]
MS + NAA 1.8 + BAP 22.5	62±7.7 [b]	56±31.5 [b]	24±21.4 [a]	59±22.2 [cd]	42±15.4 [c]	67±35.1 [b]

[*] Growth regulators in mg l^{-1}.
Numerals indicate mean values expressed in % ± SEM.
[a,b,c,d] Different letters indicate significant differences (within columns) according to Duncan's multiple comparison test ($P \leq 0.05$)

this medium presented, together with the media MS + 1.8 mg/l^{-1} NAA + 2.15 mg/l^{-1} KIN and MS + 1.8 mg/l^{-1} NAA + 22.5 mg/l^{-1} BAP, a satisfactory number of shoots derived from each explant.

Considering the good results obtained with the MS + 5 mg/l^{-1} BAP medium in all the varieties assayed, it was selected for further multiplication cycles on micropropagation reported in this paper.

Shoot multiplication cycles

After primary multiplication shoot clumps (8-20 shoots/explant) were obtained (Fig. 2a), they were separated into smaller clumps (2-3 shoots) and subcultured into liquid fresh MS medium supplemented with 5 mg l^{-1} BAP (Fig. 2b). These shoot clumps highly proliferated in successive subcultures. The shoot buds showed an increasing proliferation rate (number of shoots accumulated at the end of each multiplication cycle) during each subculture, until the fourth subculture, when it was possible to observe a constant rate (Fig. 3); the proliferation rate also depended on the genotype. Starting with one clump (2-3 shoots), 764 plants of 'Mocoi F. C. A.' and 440 plants of 'Itapé P.A.' were obtained.

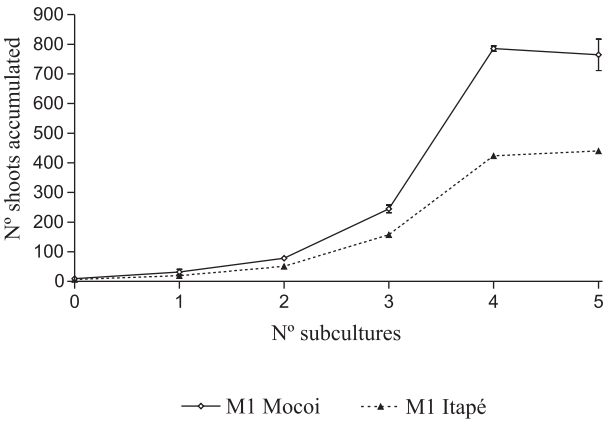

FIGURE 3. Multiple shoot production of two varieties of rice after 5 cycles (100 d) of multiplication.

TABLE 2.

Effects of six micropropagation media on the number of shoots derived from each explant in six varieties of rice, after 30 d of culture.

Culture media*	Varieties					
	IRGA 409	ITAPÉ	FORTUNA	TAIM	CT6919	MOCOI
MS	1±0.0^{a}	1±0.0^{a}	1±0.0^{a}	1±0.0^{a}	1±0.0^{a}	2±0.4^{ab}
MS + BAP 5	4±0.5^{c}	2±0.1^{abc}	2±0.6^{b}	4±0.6^{c}	5±0.8^{b}	4±0.3^{c}
MS + 2.4-D 0.2 + KIN 2.15	1±0.6^{ab}	1±0.1^{ab}	1±0.3^{a}	2±0.6^{ab}	1±0.5^{a}	2±0.4^{a}
MS + 2.4-D 0.2 + BAP 22.5	2±0.2^{b}	1±0.3^{ab}	1±0.4^{a}	3±0.3^{bc}	2±0.8^{a}	3±0.7^{bc}
MS + NAA 1.8 + KIN 2.15	2±0.2^{b}	2±0.5^{bc}	1±0.1^{a}	3±0.9^{bc}	2±0.1^{a}	3±1.4^{bc}
MS + NAA 1.8 + BAP 22.5	5±1.1^{c}	3±1.4^{c}	1±0.4^{a}	4±1.4^{c}	2±1.0^{a}	4±1.0^{c}

* Growth regulators in mg l^{-1}.
Numerals indicate mean values ± SEM.
^{a,b,c} Different letters indicate significant differences (within columns) according to Duncan's multiple comparison test ($P \leq 0.05$).

Rooting

When the shoots were transferred to the rooting medium, roots developed within a week and a profuse proliferation was observed in about three weeks (Fig. 2c). In all subcultures of both varieties, the rooting efficiency (percentage of shoots with roots) ranged from 65 to 100%.

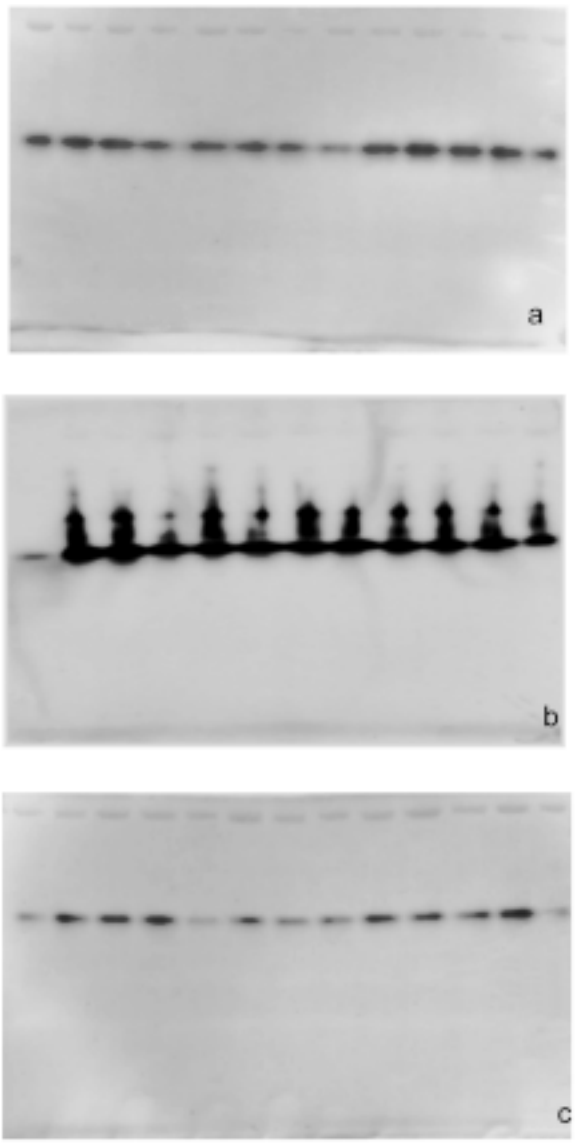

FIGURE 4. Zymograms of ADH:
a. original variety plants of 'Mocoi F.C.A.'
b. micropropagated rice plants after four multiplication cycles. Line 1 original variety, lines 2 to 12 micropropagated plants.
c. sexual progeny of the micropropagated rice plants. Line 1 original variety, lines 2 to 13 seed progeny of the micropropagated plants.

Transplant

Plantlets removed from the culture vessels and transferred to soil in benches had a survival rate of 90%. Micropropagated plants were successfully grown to full maturity (Fig. 2d). They produced seeds that were harvested and germinated in glass flasks with sterilized soil; the obtained plants were used for isozyme analysis.

Isozyme analysis

The preliminary assays performed to examine the effects of leaf position in plants of the original varieties, showed no variation in the isozyme banding patterns. Satisfactory zymograms were provided by the young leaves, which were therefore selected for the isozyme analysis.

Qualitative differences were found in the isozyme patterns of the original varieties. 'Itapé P.A.' showed two patterns for CAT, EST and PGI with a frequency of 98.3% and 1.7%. In 'Mocoi F.C.A.' coexisted two patterns for DIA with a frequency of 95.2% and 4.8%. The other isozyme systems assayed in this study presented only one band pattern.

The patterns of the different isozyme systems (ADH, CAT, DIA, EST, and PGI), for both varieties, were similar in the original variety plants and the progeny of the micropropagated plants after four multiplication cycles. However, important differences between the control and the *in vitro* micropropagated plants were detected in the zymograms of ADH (Fig. 4) and EST (not shown). *In vitro* micropropagated plants showed polymorphism for ADH bands with R_f = 0.31 and R_f = 0.38 compared to the control plants. Moreover, in EST zymograms of 'Itapé P.A.', two bands that were absent in the micropropagated plants were present in the control with R_f = 0.56 and R_f = 0.58; in the micropropagated plants, 'Mocoi F.C.A.' zymograms revealed, an additional band with R_f = 0.76 in relation to the control.

Discussion

In this work, with MS medium supplemented with 5 mg l^{-1} BAP, it was possible to obtain a high multiplication frequency for the six rice varieties evaluated, corresponding to the two subspecies. This frequency was higher than the reported by Sandhu *et al*. (1995), who worked with an *indica* rice variety, and obtained less than 10% of explants forming multiple shoots. The regeneration intensity and the proliferation rate were simi-

lar to the ones observed by Sandhu *et al*. (1995), Yoshida and Kato (1996) and Pádua *et al*. (1998). The rooting efficiency measured after four multiplication cycles remained high, allowing a successful survival after the transplant, and avoiding the delay in the growing rate that was mentioned by Yoshida and Kato (1996).

The multiplication media supplemented with 2,4-D produced calli and shoot primordia, as mentioned by Yoshida and Kato (1996). When the medium contained only BAP, it did not lead to a high necrosis of explants, as reported by Pádua *et al*. (1998), but led to a good shoot proliferation intensity.

The variety 'Mocoi F.C.A.' appeared to be the most responsive *in vitro* because it not only produced a high multiplication frequency on several micropropagation media, but was the only one with capacity to produce multiple shoots in MS lacking growth regulators.

On the other hand, we found that not only the micropropagated plants were morphologically similar to the plants of the original variety as observed by Pádua *et al.* (1998), but also their sexual progeny.

The zymograms of CAT, EST, PGI of 'Itapé P.A.' original variety plants and DIA of 'Mocoi F.C.A.' original variety plants revealed two isozyme patterns that coexisted in variable proportions. This is not the first time that such event is been reported, in fact, Galussi *et al.* (1996) also found two different electrophoretic protein models in another rice variety.

No visible morphological variation was found in the micropropagated plants that showed differences in isozyme pattern bands with respect to the controls, as reported by Noh and Minocha (1990). Davies *et al.* (1986) reported that out of 600 regenerants of *Triticum aestivum*, 13 plants revealed isozyme variation in ADH patterns, and Pereira *et al*. (1990) detected polymorphism in EST patterns of *in vitro* propagated plants of *Panicum maximum.* We also found changes in ADH and EST profiles of the micropropagated plants, but did not observe alterations in the progeny zymograms.

As it is well known that isozyme plants may vary with development and differentiation stages (Scandalios, 1974), we compared leaf tissues at the same developmental stage from the three different types of genetic material (control, micropropagated plants and their sexual progeny). So, we could suggest that the differences distinguished had a ephigenetics basis. The polymorphisms of ADH and EST patterns possibly occurred because the genetic repression mechanism that controlled gene expression was affected (Frías de Fernandez *et al.*, 1975), or because of changes due to post translational modifications (Acquaah, 1992).

We believe that the polymorphism found in the *in vitro* micropropagated plants could be only a variation during the *in vitro* procedure. No differences were found between the plants derived from the seed of the micropropagated plants and the control plants, suggesting the maintenance of the genetic stability of the micropropagated plant progeny, at least for the systems tested view. The analysis of the progeny has not been reported before for rice micropropagation based on multiple shoots methods. These findings reaffirm the necessity of the sexual progeny analysis when the aim is to accurately determine the genetic basis of the *in vitro* regenerated plants (Evans and Sharp, 1988).

In conclusion, it is possible to efficiently micropropagate rice plants, using MS medium supplemented with 5 mg l^{-1} BAP. In addition, these plants produce fertile seeds which originate plants with isozyme patterns similar to the original variety, indicating the preservation of the genetic stability in the sexual progeny of regenerated *in vitro* plants.

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