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Callus induction and plant regeneration of two Cuban rice cultivars using different seed explants and amino acid supplements

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ABSTRACT

Most of Cuban rice cultivars are classified into indica subspecies, and they are inclined to poor in vitro response. In this paper we studied the role of endosperm and amino acids on callus formation of two Cuban rice cultivars: J-104 and IACuba-28. Callus cultures were initiated from three treatments for mature seed: intact seed, embryo with scutellum but without endosperm, and endosperm alone. It demonstrated the direct incidence of endosperm on in vitro seed contamination. But the higher percentage of embryogenic calli was obtained from intact seeds, despite of 12.94 % of seed contamination. Callus formation from endosperm alone did not occur. The role of endosperm to successful callus formation from scutellum was discussed. Effect of amino acids on rice callus growth from intact seeds was examined by supplying callus formation medium with glutamine and proline, separately or in combination, in both cultivars. Callus formation of J-104 was improved considerably with 500 mg/l of proline and glutamine in the culture medium, but in IACuba-28 were not observed significant changes. The percentage of embryogenic callus and the increase of fresh weight of calli were correlated with genotype and amino acid supplement in culture medium.

Keywords: Endosperm, embryogenic calli, genotype, glutamine, proline

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INTRODUCTION

Indica rice varieties are often considered to be susceptible to tissue culture conditions and poorly responsive to transformation, most likely due to a poor callus production, somatic embryogenesis and regeneration (Lin and Zhang, 2005). The high efficiency tissue culture system for japonica rice has long been established, and it is technically robust and mature (Chu *et al.*, 1975). In indica rice, however, strong and widely applicable methods for callus culture have not been established, despite the large efforts invested in the in vitro culture. The success of all the published protocols for culturing indica rice was largely genotype-dependent, which limited the use of such protocols (Lin and Zhang, 2005). In addition, Carsono and Yoshida (2006) have described that commercial rice cultivars tended to respond badly to culture in vitro, particularly indica varieties which are recalcitrant to in vitro response.

Many factors have been investigated to improve the culture response from rice elite cultivars, including explants, media components such as alternative carbon and nitrogen sources, macro and microelement concentrations and composition, media preparation method, donor plant and growth conditions.

It has been a constant endeavour to identify suitable explants of rice to produce embryogenic calli, to maximize the callus yield. Callus formation from scutellum of rice seeds has been reported in many indica cultivars (Saharan *et al*., 2004; Kumar *et al*., 2005; Pérez Bernal *et al*., 2007). In this process, the contamination of seeds is frequent, mainly caused by the endosperm, and it certainly disturb the in vitro culture advancement. For that reason, it could be feasible through the excision of endosperm, and to use only the embryos with scutellum for callus induction, in order to reduce the seed surface and the contamination rates. But it is indispensable to know the consequences of the absence of endosperm on callus induction.

Supplementation of amino acids in culture media has been reported to enhance somatic embryogenesis in a number of monocots, such as rice. The most frequently amino acids used in callus culture are glycine, asparagine, glutamine and proline (Kopertekh and Stribnaya, 2003; Saharan *et al.*, 2004;

Abdullah *et al.,* 2005). Amino acids provide a source of reduced nitrogen, which is readily metabolized by plant cells, stimulating faster cell growth and development. Differential responses to organic nitrogen sources indicate the requirement of specific amino acids for specific events during *in vitro* morphogenesis. Therefore, the additional amino acids appear to have the potential to enhance to some extent the roles of suitable nitrogen sources (Sarker *et al.,* 2007).

Most of Cuban rice cultivars are classified into indica subspecies, and they are inclined to poor *in vitro* response. More attention is required to improve tissue culture conditions of two national important varieties: J-104 and IACuba-28. In this paper we evaluated different seed treatments for embryogenic callus formation of both cultivars. The composition of medium for callus culture, described in previous studies (Pérez-Bernal *et al,* 2002; 2007), was modified by addition of amino acids. Here we demonstrated the role of endosperm and amino acid supplement to improve the embryogenic callus formation from mature seeds of J-104 and IACuba-28 rice cultivars.

MATERIALS AND METHODS

Seed sterilization

Mature seeds of indica rice cultivars IACuba-28 and J-104 were collected from Rice Experimental Station "Sur del Jíbaro", La Sierpe, Cuba. Seeds were manually dehusked and washed in 70% ethanol for 1 minute. Sterilization of seeds was carried out using a solution of sodium hypochlorite with 2.5% active chlorine. After 20 minutes the solution was discarded, seeds were thoroughly washed 4-5 times with sterilized water and placed on absorbent paper to remove residual water.

Seed treatments for callus formation

Sterilized seeds of two cultivars were divided into three independent groups. The first group included intact seeds, in the second group the endosperm was eliminated under stereoscopic microscope using a scalpel,

getting only the embryo with scutellum, and in the third group only endosperms were preserved.

All groups of seeds were placed into Petri dishes containing callus formation medium (N62), which consisted of salts and vitamins of N6 (Chu *et al*., 1975), sucrose 30 g/L, 2,4-D 2.5 mg/L, casein hydrolysate 1 g/L and Phytagel® 3 g/L; pH was adjusted at 5.7. Cultures were maintained at 27±1ºC in dark for 21 days.

Inclusion of proline and glutamine in callus formation medium

Sterilized intact seeds of two cultivars were planted on callus formation medium described previously, but including different treatments with proline and/or glutamine, as shown in Table 1. Callus culture was carried out at 27±1ºC in dark for 21 days.

Table 1. Culture media used for rice callus formation with proline and glutamine.

Culture media	Proline and Glutamine treatments
N62 (Control)	Without proline and glutamine
N62P-100	Proline 100 mg/l
N62P-500	Proline 500 mg/l
N62G-100	Glutamine 100 mg/l
N62G-500	Glutamine 500 mg/l
N62PG-100	Proline 100 mg/l, Glutamine 100 mg/l
N62PG-500	Proline 500 mg/l, Glutamine 500 mg/l

Embryogenic callus evaluation

Distinction between embryogenic and non-embryogenic callus was established on the basis of callus morphology, using a stereoscopic microscope for the observation of pro-embryogenic and globular structures. After 21 days of culture, the number of embryogenic calli was recorded for each cultivar. These data were expressed as percentage of embryogenic calli in relation to the total number of calli. The increase of fresh weight of calli was determined using random samples of 100 calli per treatment with proline and/or glutamine.

Statistical analysis

Two way analysis of variance (ANOVA), completely randomized, and multiple comparisons of means by Student-Newman-Keuls Test ($p \leq 0.05$) were used to analyze the percentage of embryogenic calli and the increase of their fresh weight, with 14 replicates per cultivar. For each treatment 140 calli were used, planted in 10 per Petri dish. Data were processed using version 11.5 of SPSS program.

RESULTS

Seed treatments

Three weeks after placing the seed in callus formation media, we analyzed the results of seed sterilization process. Percentage of contamination in intact seeds (12.94%) was two fold higher than seeds without endosperm (6.47%). The group of endosperms exhibited a 12.06% of contamination, which was similar to intact seeds. The most frequent contaminating agent was fungi.

It was found that callus formation from intact seeds was more effective in relation to the other treatments. The percentage of embryogenic callus formed from intact seeds was more than 4 times higher compared to the percentage obtained from seeds without endosperm. Callus formation from endosperm alone did not occur. Genotype has no effect on this result: there were not significant differences ($p \leq 0.05$) between J-104 and IACuba-28 (Figure 1).

Embryogenic calli formed from intact seeds were qualitatively better. They have a glossed and compact aspect, characterized by their yellow colour, with proembryogenic and globular-translucent structures (Figure 2A). Non-embryogenic calli are of wet aspect, scarce growth and white in colour (Figure 2B). Endosperm of intact seeds was softened four days after seed placing on callus formation medium. Every calli formed from seeds without endosperm were undersize and brownish. A major proportion of non-embryogenic calli was observed in respect to embryogenic ones. J-104 and IACuba-28 showed similar behaviour in all treatments.

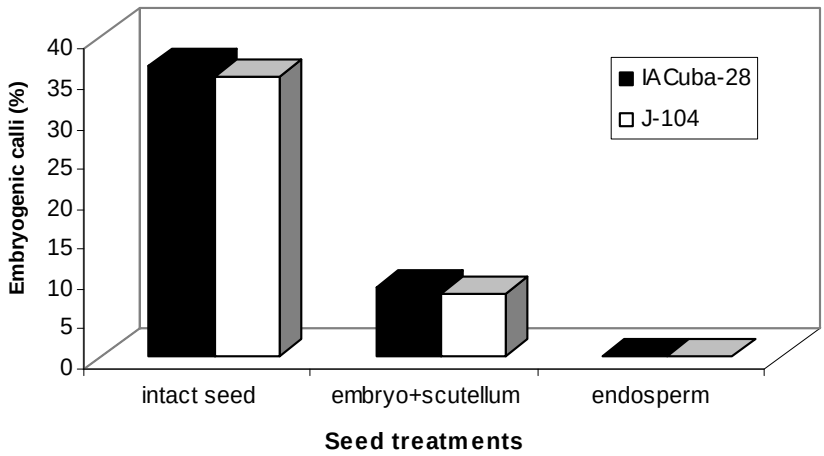

Figure 1. Percentages of embryogenic callus formed from different seed treatments of two indica rice cultivars, IACuba-28 and J-104, after 21 days of *in vitro* culture. Means with different letters indicate significant differences between treatments (Student Newman Keuls Test, *pd"0.05*).

A

B

Figure 2. Calli induced from intact mature seeds of rice. (A) Callus with globular somatic embryos. (B) Non-embryogenic calli.

Effect of proline and glutamine on callus formation

Notable differences in the formation of embryogenic callus were distinguished in J-104 rice cultivar between treatments with proline and glutamine, in respect to control N62. The maximum percentage of embryogenic calli was obtained with N62PG-500, and there were not significant differences ($p \leq 0.05$) between treatments with proline or glutamine at 100 mg/l and 500 mg/l (Figure 3). IACuba-28 did show significant differences neither in embryogenic callus percentages nor in fresh weight growth when amino acids were added to culture medium.

Proline and glutamine promoted the increase of fresh weight in J-104 rice calli, but not in IACuba-28 (Figure 4). Most J-104 rice callus random samples augmented their weight more than 1.22 g when proline or glutamine or both were included in callus formation medium. The best result was obtained when calli were cultivated on N62PG-500, which exhibited a 3.14 fold increase in fresh weight in respect to calli cultivated on medium without amino acids. Growing of numerous globular somatic embryos was the most significant effect visualized over calli cultured on media N62PG-100 and N62PG-500. Nevertheless, IACuba-28 rice calli did not increase their globular structures in relation to control without amino acids.

DISCUSSION

The effect of endosperm on seed contamination was confirmed in the present study. Due to this, it could be feasible the excision of endosperm and the use of the embryo with scutellum for callus induction, in order to reduce the seed surface and the contamination rates. However, the absence of endosperm affects considerably the callus formation from seeds.

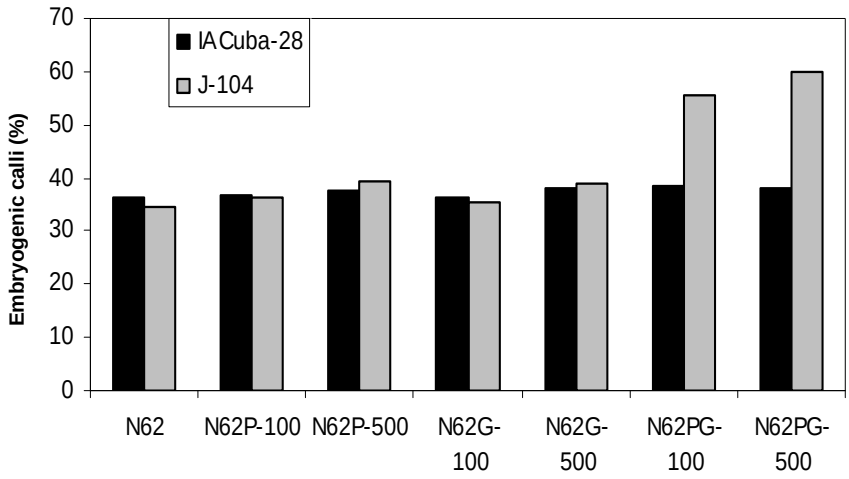

Figure 3. Percentages of embryogenic calli of indica rice cultivars, IAcuba-28 and J-104, from intact seeds cultivated with different treatments of proline and glutamine. Evaluation was made at 21 days of callus culture. Means with different letters indicate significant differences between treatments (student newman keuls test, *pd*”0.05).

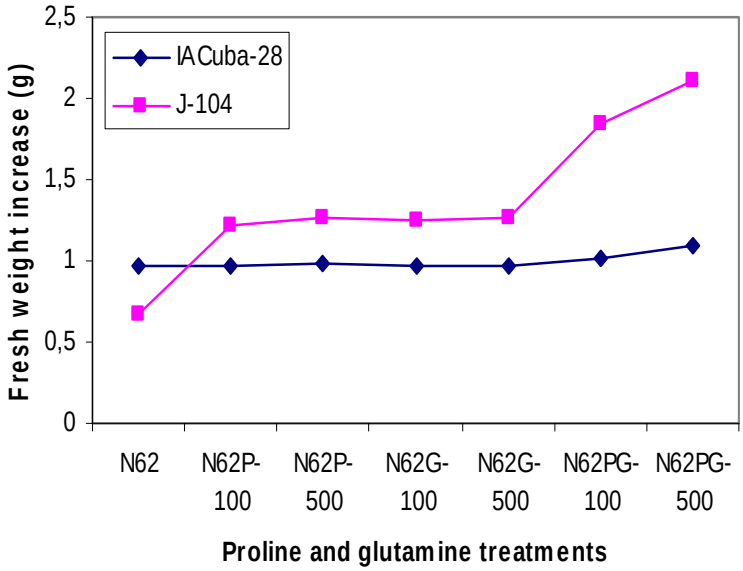

Figure 4. Increase of fresh weight of calli of IAcuba-28 and J-104 rice cultivars, from intact seeds, on different culture media with proline and/or glutamine. Evaluation was made at 21 days of callus culture. Fresh weight was determined using random samples of 100 calli per cultivar in each treatment.

In fact, callus formation invariably developed from the scutellar region of seeds of both indica rice cultivars, independent of genotype, according with results of Saharan *et al*. (2004). Swelling of scutellum of rice seeds, observed after 4 days of culture on callus formation medium, was followed by development of primary calli from the scutellum and proliferation of proembryogenic and globular structures (Saharan *et al*., 2004; Kumar *et al*., 2005). Krishnan *et al*. (2001) explained that scutellar cells store large amounts of protein, phytin and lipids; the scutellar epithelial cells secrete enzymes into the starchy endosperm to digest the macromolecules, and reabsorbs low molecular weight substances to be transported to the embryo, that could allow the physiological events on in vitro culture seeds. Storage parenchyma of endosperm expires when were removed their reserves. That is a possible reason of endosperm softness four days after seed placing on callus formation medium.

Callus formation took place successfully from scutellum of IACuba-28 and J-104 rice seeds, however, from endosperm alone it did not occur. Plant regeneration from endosperm-derived calli is impossible under assayed conditions. For that reason, regeneration of triploid plants, coming from endosperm, can be discarded.

In the case of calli formed from seeds with embryo and scutellum, but without endosperm, it was obtained low percentage of embryogenic callus compared to intact seeds, supporting the importance of endospermic reserves for all physiological events of seeds. The endosperm is primarily a storehouse of starch and some proteins. When seeds are placed on culture medium with high amount of water, seed imbibition occurs followed by the mobilization of reserves. The first step in the mobilization of reserves in rice seed is the decomposition of amyloplast envelopes in endosperm cells adjacent to scutellum, causing the separation of the starch granules, which became smaller in size and were covered with high-viscosity materials assumed to be soluble polysaccharides (Zakaria *et al.* 2000). Obviously the starchy endosperm can also make a significant contribution of nutritional bases to callus development, besides media constituents.

The exogenous strong auxin (2,4-D) in culture medium blocks the normal seed germination process, allowing cell dedifferentiation and callus induction. But endosperm reserves can be assimilated anyway. Consumption of reserves was evidenced when endosperm turned soft in a short time after seed placing on culture medium.

Amino acids provide a source of reduced nitrogen and they are commonly included as organic supplement in rice tissue culture. In this study, the inclusion of proline and glutamine in callus formation medium increased considerably the percentages and the growing of J-104 embryogenic calli, and did not produce significant changes in IACuba-28 calli. Requirements of nitrogen supplement were genotype-dependent for callus formation. There was genotype-medium interaction for inducing calli with an organized embryogenic structure and for increasing fresh weight of calli.

It is clear that J-104 rice cultivar needs high quantities of organic nitrogen for callus formation. Supplementing of N62 medium with 500 mg/l of proline and glutamine not only increased J-104 callus growth but also improved embryogenic callus formation. The effect produced by amino acid mixture suggested that amino acid interaction was involved in this instance. The addition of a combination of amino acids was also beneficial for the induction of somatic embryos in wheat (*Triticum aestivum*) (Trottier *et al*., 1993) and barley (*Hordeum vulgare*) (Ouedraogo *et al.*, 1998). Due to the complexity of amino acid metabolism, future detailed research efforts should investigate the properties of amino acids combination that do play a major role in tissue culture of indica rice.

It was found that proline and glutamine were highly effective at concentration of 500mg/L, according with the experimental conditions reported by Brisibe et al. (2000), using two lines of wheat callus for the productive regeneration of green plantlets, maintained on media with glutamine and proline. Sarker *et al* (2007) reported lower concentrations of asparagine for induction of somatic embryos from immature zygotic embryo derived calli of wheat. Karamanos (1995) has discussed the role of proline as an energy, carbon and nitrogen source which enhances tissue recovery on the

relief of stress. Other authors suggested that amino acids could promote changes in gene expression (Sahrawat and Chand, 2001).

Great increase of globular structures and fresh weight of J-104 rice calli, give reasons to consider the genotype-dependent role of *in vitro* amino acid supplement in callus culture media. That confirms the genotypic effect as a significant factor controlling the response to *in vitro* culture conditions. The major influence on tissue-culture response appears to be genetic, with culture requirements varying between species and cultivars. Genotype and culture response were correlated and significantly differed in inducing high quality of calluses depending on medium used. This analysis enabled to focus such *in vitro* parameters that need to be improved in the genotypes of choice, specifically J-104, which is the most important commercial rice cultivar in Cuba. These observations would be useful for tissue-culture based research and for crop improvement, particularly for genetic transformation.

CONCLUSIONS

Endosperm is essential for successful embryogenic rice callus formation from mature seeds, regardless of its effect on *in vitro* seed contamination.

Combination of proline and glutamine, both at 500 mg/l in callus formation medium, can increase embryogenic masses in J-104 rice calli, but do not produce changes in IACuba-28 calli.

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