

# Characterization of storage proteins and anther culture for the development of high nutritional quality rice genotypes

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## ABSTRACT

Rice (*Oryza sativa* L.) is economically important worldwide since it is the cereal most highly consumed by humans; so, it is important to improve its nutritional properties. This paper reports the results of the biochemical characterization of storage proteins in seeds of segregating generations (F2 and F4) from (Morelos A-92) [MA92; Indica] x Kosihikari [Kosi; japonica] and Kosi x MA92) rice hybrids as well as induction of androgenesis. The highest total protein content (10.77%) was detected in Kosi x MA92 (F2). The F4 generation from Kosi x MA92 genotype showed the highest levels of albumin, globulins, and prolamins as well as the lowest percentage of glutelins (21.48%). The electrophoretic profile of globulins isolated from Kosi x MA92 hybrid (segregant-F4) showed 13 bands with 13-110 kDa molecular weights while glutelins just three bands (13, 16, and 21 kDa). Androgenesis was induced in the N6 culture medium, supplemented with ANA, 2.0; KIN, 1.0; glycine, 2.0 and glutamine, 500 mg/L, maltose 5%, and agar 8 g/L. The conversion of embryogenic structures into plants was performed in Murashige & Skoog (MS) supplemented with AIA, 0.5; ANA, 0.5, and KIN, 2.0 mg/L, sucrose, 30 and agar, 8 g/L.

**Keyword:** storage proteins, hybrids, androgenesis, genetic improvement, *Oryza sativa* L.

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RESEARCH

## RESUMEN

**Caracterización de las proteínas de reserva y cultivo de anteras para el desarrollo de genotipos de arroz de alta calidad nutricional.** El arroz (*Oryza sativa* L.) es de gran importancia económica mundial, pues es el cereal de mayor consumo humano, por ello es importante buscar alternativas para mejorar sus propiedades nutricionales. En este trabajo se muestran los resultados sobre la caracterización bioquímica de las proteínas de reserva en semillas de generaciones segregan (F2 y F4) de híbridos (Morelos A-92 [MA92; indica] x Kosihikari [Kosi; japónica] y Kosi x MA92) de arroz, así como la inducción de androgénesis. El contenido más alto de proteínas totales (10.77%) se detectó en Kosi x MA92 (F2). El genotipo Kosi x MA92 generación F4 presentó los niveles más altos de albúminas, globulinas y prolaminas, y el menor porcentaje de glutelinas (21.48%). Se obtuvieron resultados relevantes en el perfil electroforético de las globulinas del híbrido Kosi x MA92 (segregante F4), con 13 bandas cuyos pesos moleculares estuvieron entre 13 y 110 kDa, y en el perfil electroforético de las glutelinas solo hubo tres bandas de 13, 16 y 21 kDa. La androgénesis se indujo con el medio de cultivo N6 adicionado con ANA, 2.0; KIN, 1.0; glicina, 2.0 mg/L y glutamina, 500 mg/L; maltosa 5% y agar 8 g/L. La conversión de estructuras embriogénicas a plantas se hizo en el medio de cultivo Murashige & Skoog (MS), suplementado con AIA, 0.5; ANA, 0.5; KIN, 2.0 mg/L; sacarosa, 30 mg/L y agar 8 g/L.

**Palabras clave:** proteínas de reservas, híbridos, androgénesis, mejoramiento genético, *Oryza sativa* L.

## Introduction

Rice culture is economically important worldwide since it is the cereal most highly consumed by humans. Each rice grain contains 90% starch, and 10% protein. Rice is important from the nutritional point of view, due to the high quality of its protein which contains 60% essential amino acids, higher than other widely consumed grains such as corn and wheat with 40 and 43% respectively [1]. Storage proteins in rice seeds are composed of albumins (5%), globulins (10%), prolamins (5%), and glutelins (80%) [2]. The biochemical characterization of storage proteins allows the identification of genotypes that show important nutritional characteristics, for instance: the Index of Essential Amino Acids (IEA), which is determined by the amount of amino acids needed by the human body that can not be metabolized so that direct ingestion is necessary. This index is directly proportional to globulin fraction content which represents 75-95% of total protein in legume species

[3]. Traditionally rice breeding has been focused on improving agro-economic characteristics, increasing yield per surface unit, inducing resistance to plant pathogens or by increasing grain yield. Currently new challenges have arisen; the demand for nutraceutical products is growing, which may require improve nutritional properties and to reduce cereal specific allergenic problems [4]. The development of genotypes with high globulin content is one of the alternatives to improve the nutritional properties of rice, because of its direct relation with IAE. Genotypes with such characteristics may be subject to anther culture. This technique has shown to be useful tool in genetic improvement which has the advantage of fixing characteristics faster (50%) than that required by traditional breeding methods.

The integration of methodologies for genetic improvement, anther culture, biochemical and molecular characterization, allows the generation of rice varie-

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ties with a high nutritional value and without allergens for specific markets. Which will increase profits. It is ever reported that the area devoted to rice has decreased due to the high culture cost. The purpose of this research was for the biochemical characterization of storage proteins from rice hybrids segregating generations to obtain genotypes with the needed biochemical characteristics (higher globulin content) as well as to establish a micro-propagation methodology by culturing anthers from selected hybrids.

## Materials and methods

### Biological material

Seeds of F2 and F4 segregating generations from rice hybrids were used. They were obtained from direct crosses of Koshihikari (Japónica) x Morelos A 92 (Indica) (Kosi x MA92) and MA-92 x Kosi. Their parents were used as a control. These genotypes are part of the germplasm bank of Zacatepec-INIFAP experimental field in Zacatepec, Morelos, Mexico.

### Determination of total protein content

Seeds were manually husked and milled in a Teckmar A-10 (Teckmar, USA) analytical mill and the flour obtained was passed through an 80 mm diameter sieve to homogenize particle size. Total Nitrogen content of each hybrid was determined by the Kjeldahl [5] method. A 5.85 conversion factor, specific for cereals, was used for the calculation of total protein percentage.

### Storage proteins

Storage proteins (albumins, globulins, prolamins, and glutelins) were obtained from flour based on solubility. The Osborn method, modified by Texeira and Ferreira [6] was used for this purpose. A 1:10 (m/V) flour-extracting solution was used for protein fractionation. The process started with the extraction of albumins by adding 10 mM CaCl<sub>2</sub> and 10 mM MgCl<sub>2</sub>, stirring constantly for 4 hours at 4 °C. Followed by centrifuging at 10 000 g for one hour. The supernatant was dialyzed against water for 12 hours and then stored at 4 °C; the precipitate was resuspended in a Tris-HCl 100 mM solution, NaOH 10%, EDTA 10 mM and EGTA 10 mM solution, pH 7.5, kept at constant shaking at 4 °C for 4 hours and centrifuged at 10 000 g for an hour, the supernatant was dialyzed against water. Globulins were obtained; the precipitate was resuspended in 75% ethanol for the extraction of prolamins; it was shaken for 12 hours and then centrifuged at 10 000 g for an hour. The supernatant was dialyzed against 1% acetic acid. Finally the precipitate was subject to a last extraction to obtain glutelins by solubilizing them in an HBO<sub>3</sub> 50 mM solution, β-Mercaptoetanol 1% (V/V) SDS 1%, pH 10, shaken for 4 hours at room temperature then centrifuging at 10 000 g for an hour. The supernatant was dialyzed against water. Protein fractions and the residual flour were stored at 4 °C for subsequent characterization.

### Determination of nitrogen content and storage proteins

Once four protein fractions (albumins, globulins, prolamins, and glutelins) and residual flour were obtained, protein content was quantified by the Kjeldahl.

### Electrophoretic profiles

The biochemical characterization of storage proteins was made by electrophoresis (SDS-PAGE) [7] in denaturing conditions in 10% polyacrylamide gels. Samples of 15 mL of each fraction per lane were loaded and 20 mA per gel were used. After the run each gel was fixed with 12% trichloroacetic acid and stained with Coomassie blue R-250. A kit of pre-stained standards [myosin (205 kDa), β-phosphorilase (97 kDa), albumin from bovine serum 66 (kDa), ovoalbumin 45 (kDa), carbonic anhydrase (29 kDa), and α-lactoalbumin (14 kDa)] of Bio-Rad, CA, was used as reference for the determination of molecular weight (MW).

### Statistical analysis

A one-way variance analysis (ANOVA) was made and Turkey test was used for mean comparison with  $\alpha = 0.05$ .

### Anther culture

#### Anther collection and disinfection

Panicles of MA92 x Kosi and Kosi x MA92 hybrids were collected in the field when pollen grains were at a mid-late uninucleated stage [8]; given a cold pre-treatment at 6 °C for 10 days, disinfected in aseptic conditions and placed in a NaOCl solution (1.2% V/V for 10 minutes). Then they were thoroughly rinsed with sterile distilled water.

#### Induction of the embryogenic callus

Anthers were extracted from the panicle [9] and placed in 60 x 15 mm disposable Petri dishes, which contained N6 [10] culture medium, supplemented with: ANA 2.0 mg/L, KIN 1.0 mg/L, glycine 2.0 mg/L, glutamine 500 mg/L, maltose 5% y agar 8 g/L [11].

An average of 208 MA92 x Kosi and 423 de Kosi x MA92 genotype anthers were planted.

The percentage of embryogenic callus induction was obtained by counting the number of anthers with calluses and dividing by the total number of anthers planted; the result was multiplied by 100. The conversion of embryogenic structures in plants was performed in the MS culture [12] supplemented with AIA 0.5 mg/L, ANA 0.5 mg/L, KIN 2.0 mg/L, sucrose 30 g/L, and agar 8 g/L [13].

## Results and discussion

### Content of total proteins

Table 1 shows percentages of total proteins obtained for each rice genotype. The proteins content increased from 7.59% in MA92 to 9.67-10.77% in hybrids (F2 and F4 segregating generations). Values were slightly higher when the female parent was Kosi (japonica). The increase in total protein could be due to the difference in genetic background in the japonica subspecies rice variety.

### Determination of percentage of storage proteins

Table 2 shows the percentage (%) of storage proteins (albumins, globulins, prolamins and glutelins) and residual flour, obtained from the rice genotypes.

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Table 1. Total protein content in various rice genotypes

Rice genotypes	Total protein (%)*
Kosi x MA92 F2	10.77
Kosi x MA 92 F4	10.37
MA92 x Kosi F2	10.64
MA92 x Kosi F4	9.67
Kosi	9.78
MA92	7.59

\* Percentage N x 5.85, average of three repetitions

Kosi x MA92 genotype generation F4 showed the highest levels of albumins, globulins and prolamins, as well as the lowest percentage of glutelins (21.48%). Compared to the F2 segregant from the same hybrid, this genotype showed a 50.4, 31.7, and 22.7% increase in the content of globulins, prolamins and albumins respectively while the glutelin fraction decreased 56.1%.

On the other hand, genotypes MA92 x Kosi and Kosi x MA92 (F2 segregant) showed the highest percent of glutelins (49.38 and 48.89% respectively).

These results show that rice genotypes with different genetic characteristics and increasing nutritional value may be achieved of thus generating differentiated genotypes that may be destined to special market niches.

### Structural characteristics of storage proteins

Globulin and glutelin electrophoretic profiles show similarities in the number of subunits as well as in molecular weights in the rice genotypes.

Figure 1 shows the electrophoretic pattern of globulins for each rice genotype; lane one corresponds to Kosi x MA92 generation F4 in which globulin bands are more defined and intense, compared with the other genotypes, which may indicate that there was a higher accumulation of this protein fraction in that genotype. On the other hand, in the molecular weight of subunits composing globulins of rice genotypes were similar to those reported by Fukushima [14]. Bands observed co-respond to molecular weights between 11 and 112 kDa, being those of 20, 27, 55, 80 kDa in a higher proportion.

The electrophoretic profile of glutelin fractions of the five rice genotypes revealed molecular weights for subunits at a 13-100 kDa interval (Figure 2), with those of 14, 18, 22, 34 and 57 kDa in a higher proportion. Molecular weights similar to these subunits [2] have been reported in other rice varieties.

Finally, a significant decrease in the glutelin fraction took place in the Kosi x MA92 genotype, F4 generation whose electrophoretic profile shows three bands of 13, 16, and 21 kDa. Likewise it showed the highest content of globulins with 13 bands of molecular weight between 13 and 110 kDa.

### Induction of the embryogenic callus

The callus obtained from the Kosi x MA92 hybrid was friable creamy white with the presence of segregates of about 1 mm and a hard consistency. The

Tabla 2. Porcentaje de proteínas de reserva en cada genotipo

Storage proteins	KOSI x MA92 F2 %	KOSI x MA92 F4 %	MA92 x KOSI F2 %	MA92 x KOSI F4 %	KOSI %
Albumins	11.39 b	14.74 a	6.91 d	8.11c	7.86 c
Globulins	14.69 c	29.64 a	19.49 b	21.25 b	19.23 b
Prolamins	17.24 b	25.24 a	16.64 b	17.18 b	15.17 b
Glutelins	48.89 ab	21.48 c	49.38 a	41.97 b	44.34 b
Residual flour	7.56 cd	7.82 bc	7.38 cd	10.57 a	9.86 ab

Values are reported in percentage and represent the average of three repetitions. The results were converted into natural logarithm (Ln) for statistical analysis. Equal letters indicate no significant difference  $\alpha=0.05$ .

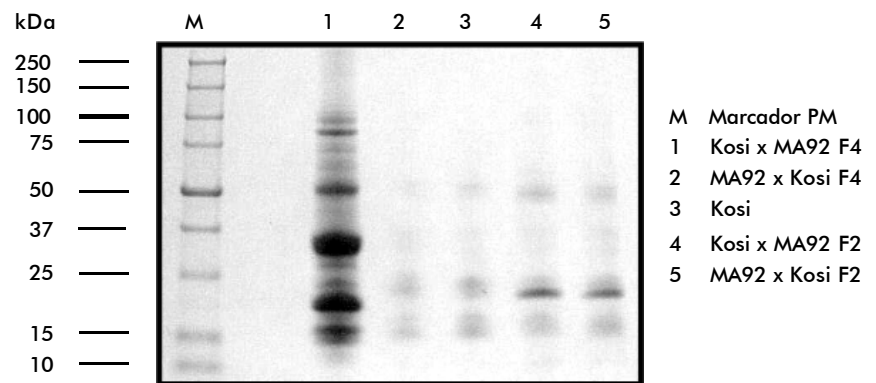


Figure 1. Electrophoretic pattern (SDS-PAGE) of globulins of different rice genotypes.

callus from MA92 x Kosi hybrid was similar in aggregate size and consistency, but white in color with a velvet appearance (Figure 3). Those characteristics correspond to embryogenic callus according to what is reported for other rice genotypes, from diverse explants, and even from anthers [15].

The embryogenic callus was transferred to a conversion medium and green embryogenic structures developed. The embryogenic structures became vigorous shoots. Those shoots were transferred to an SM medium, supplemented with indolacetic acid (1.5 mg/L) and benzylaminopurine (0.2 mg/L) where they formed an abundant radical system (Figure 4).

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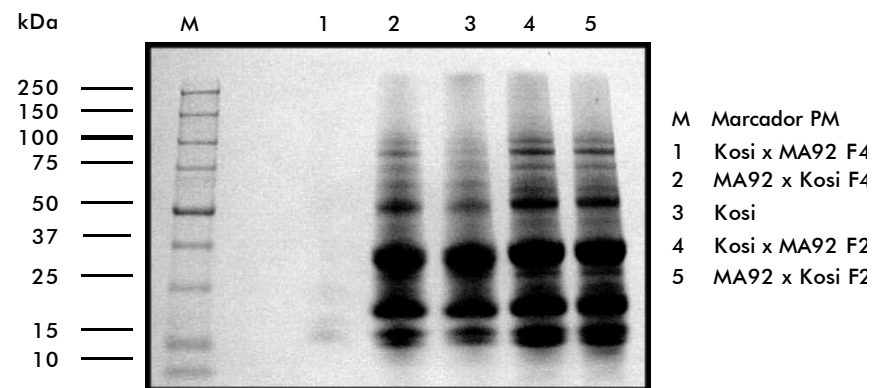


Figure 2. Electrophoretic pattern (SDS-PAGE) of glutelins of different rice genotypes.

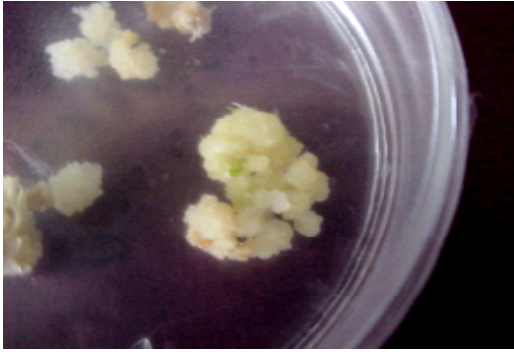


Figure 3. Embryogenic callus obtained by anther culture.

## Conclusions

The results of the present study revealed that a significant decrease (more than 50%) of the glutelin fraction took place in the Kosi x MA92 rice hybrid from the F4 segregating generation, as well as an increase in the content of globulins, prolamins, and albumins, which was higher in fractions of globulins (more than 50%). This demonstrates the possibility of obtaining improved rice genotypes to increase nutritional value. These biochemical characteristics may be set in the plants in a shorter time using anther culture technique for producing plantlets of MA92 x Kosi and Kosi x

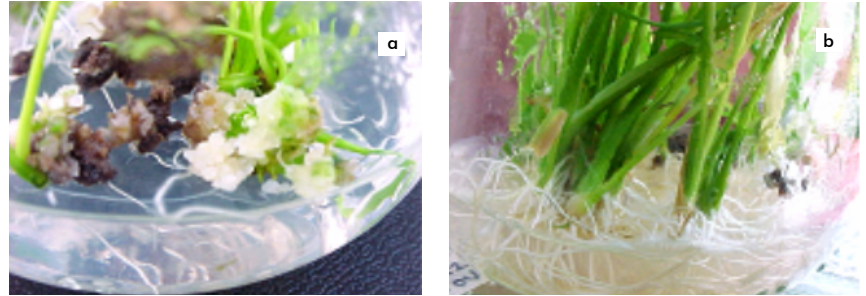


Figure 4. a) Conversion embryogenic structures in plants. b) Development of roots in shoots obtained from embryogenic restructures.

MA92 hybrids. The integration of methodologies for traditional genetic improvement, anther culture and biochemical characterization allows to obtain differentiated rice varieties to meet industry and consumer needs as well as to increase rice production profits.

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