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RESEARCH

Obtaining double haploid (DH) populations in crossbreeding of rice cultivars (*Oryza sativa* L.) with indica and japonic subspecies and evaluation of their tolerance to drought

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ABSTRACT

The drought causes negative effects on the productivity of the plants and constitutes a potential danger for the food sustainability of the population. One of the most difficult tasks for rice breeders is to increase tolerance to salinity and drought, in addition to achieving high and stable yields. In the genetic improvement program of this precious grain, different improvement methods are used, where biotechnological techniques have been used, using the *in vitro* anther culture to shorten the time for obtaining haploid lines, which will be subsequently evaluated in conditions of water deficit. For these reasons, this work was aimed to obtain double haploid lines through *in vitro* anther culture of F2 hybrid populations, and evaluate them under water deficit conditions. Crosses were made between five drought-resistant cultivars and six cultivars of good agronomic performance. Anthers of F2 plants were cultured *in vitro* to evaluate callus formation in three liquid media: N6m, NL and N6-1. Then, agronomic characters and tolerance to water deficit were evaluated in the first generation of isogenic lines obtained. Double haploid lines that combined drought resistance and good agronomic traits were evaluated under field conditions without watering, only with water passes as the plant required. The use of the *in vitro* anther culture technique showed the high dependence on genotype and culture medium. The highest values of callus formation were achieved in the NL culture medium. New double haploid lines resistant to water deficit and showing high agricultural yield were obtained.

Keywords: rice, anther culture, genetic improvement, double haploid variants

RESUMEN

Obtención de poblaciones doble haploides (DH) en cruzamiento de cultivares de arroz (*Oryza sativa* L.) con subespecies indica y japónica, y evaluación de su tolerancia al déficit hídrico. Una de las tareas más difíciles para los mejoradores de los cultivos de arroz es lograr incrementar la tolerancia a la salinidad y la sequía, además de lograr mayores producciones. En el programa de mejoramiento genético del arroz se emplean diferentes métodos, y el uso de técnicas biotecnológicas mediante el cultivo in vitro de anteras, el cual acorta los tiempos para la obtención de líneas haploides, las cuales se evalúan en condiciones de déficit hídrico. Por lo tanto, el propósito de este trabajo fue la obtención de poblaciones hibridas F2 mediante la aplicación de este método, y su posterior evaluación en condiciones de sequía. Se efectuaron cruzamientos entre cinco cultivares resistentes a la sequía y seis de buen comportamiento agronómico, y las anteras de las plantas F2 fueron cultivadas in vitro para evaluar la formación de callos en tres medios líquidos: N6m, NL y N6-1. A la primera generación de las líneas isogénicas obtenidas se le evaluó los caracteres agronómicos y su tolerancia al déficit hídrico. Las líneas dobles haploides con valores positivos en ambos parámetros fueron evaluadas en condiciones de campo sin aniego, solo con pases de agua según la planta lo requería. La utilización de la técnica de cultivo in vitro de anteras mostró la alta dependencia del genotipo y del medio de cultivo empleado. Con el medio NL se alcanzaron los valores más altos para la formación de callos. De esta forma, se obtuvieron nuevas líneas dobles haploides resistentes al déficit hídrico y con alto rendimiento agrícola.

Palabras clave: arroz, cultivo de anteras, mejoramiento genético, variantes dobles haploides

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Introduction

The world rice production has reached 701 million tons of husk rice in average yearly during the last ten years, and a stable 150-162 million ha plantation area [1].

It is among the top consumed food in Latin America and an essential food with 60 kg consumed per capita [1] in Cuba. However, the national production in this country does not surpass the 50 % of the demand, obligating to import significant amounts to satisfy the dietary needs [2]. Despite the rice crop national variety policy comprising more than ten cultivars of the species, all of them show limitations to reached their expected production potential. In fact, crop yields reach 3.6 ton/ha, below the world marks [3]. It is mainly caused, for instance, by the influence

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of the climate, the insufficient exploitation of the optimal seeding season, deficient crop management practices, the exhausting of soil components, the impact of pests, soil salinity and dryness, among others [1]. Dryness is one of the most significant since there are more than 100 000 areas under cultivation without enough irrigation sources [4, 5].

Currently, nearly 80 % of all the water available in the country are used for agriculture, and 40 % of produced foods worldwide are cultured in irrigated areas [6].

In this setting, submerged rice demands 16 000 m^3/ha on each cultivation cycle [1]. Current environmental changes indicate the possibility of a future increase of dryness. Therefore, it is necessary to seek for feasible alternatives for a more efficient use of cultivation water in order to reduce the irrigation needed due to its limited availability [5-7].

In Cuba, a significant alternative to try to circumvent such limitations comes from obtaining rice cultivars of low water consumption by genetic improvement procedures. In fact, improved yields have been reached almost exclusively by using traditional crop improvement procedures.

Noteworthy, biotechnological methods provide new tools for improving the existing cultivars more suitable and adapted to stressing culture conditions and providing potentially higher production yields [8]. For instance, cell and haploid tissue culture guarantee achieving homozygosis in a single generation, thereby reducing considerably the improvement cycle, something feasible through anther culture [8-11]. Two new cultivars were recently obtained with this method and included in the Registry of Agricultural Varieties of the Cuban Ministry of Agriculture. They show high production potential, good cooking properties, resistance to drought and to the incidence of piriculariosis, despite their limited extension to the cooperative agricultural sector and not to the industrial farming. Moreover, the use of anther culture allows the generation of rice cultivars in just 5 out of the 10-year period when using traditional breeding methods [8, 12]. Therefore, this work was aimed to obtain double-haploid rice variants through *in vitro* anther culture and evaluate them for drought resistance in F2 generation plants.

Materials and methods

Hybridizations of selected cultivars resistant to drought and showing good agronomic performance

Twenty-four hybridizations were made with selected rice progenitor cultivars [13]. For this, a Topcross statistic design was followed, and each cultivar showing good agronomic performance was used either as female or male progenitor cultivar for crossing with drought resistant cultivars (Table 1). Cultivars were staggered planted under semi-controlled conditions considering their cycle and after 30 days they were transplanted to field conditions. Phytotechnical management was done as recommended by the agricultural cultivation standard procedures [1].

Female progenitor panicles were collected when they emerged 50-60 % and anthers were extracted with a needle. Male progenitor panicles were collected fully emerged and shaken over the emasculated female panicles for pollination. This procedure was done for five days, and then female panicles were completely covered with semi-translucent paper and properly identified with the cross breeding information and date.

Table 1. Hybridization made with progenitor cultivars to generate drought-resistant cultivars

No.	Crossbreeding (female/male)	No.	Crossbreeding (female/male)
1	Amistad'82/C4 153	13	C4 153/Amistad'82
2	Amistad'82/IR 20	14	IR 20/Amistad'82
3	Amistad'82/IR 36	15	IR 36/Amistad'82
4	Amistad'82/Perla de Cuba	16	Perla de Cuba/Amistad'82
5	INCA LP 10/C4 153	17	C4 153/INCA LP 10
6	INCA LP 10/IR 20	18	IR 20/INCA LP 10
7	INCA LP 10/IR 36	19	IR 36/INCA LP 10
8	INCA LP 10/Perla de Cuba	20	Perla de Cuba/INCA LP 10
9	IR1529-430/Amistad'82	21	Amistad'82/IR1529-430
10	Moroberekan/Amistad'82	22	Amistad'82/Moroberekan
11	INCA LP 10/Amistad'82	23	Amistad'82/INCA LP 10
12	IR 1529-430/INCA LP 9	24	INCA LP 9/IR 1529-430

Afterwards, mature seeds resulting from crossbreeding were harvested. They were disinfected with 1.5 % hypochlorite for 1 min, and washed several times with distilled water. Subsequently, they were placed in Petri dishes containing distilled water and incubated at 30 ºC. Approximately 10 days after germination they were transplanted to pots containing a mix (1:3) of Gley Nodular Ferruginous petroferric soil [14] and curated bagasse, respectively.

After thirty days, seedlings were transplanted under field conditions into groves separated 40 cm, with 30 cm between plants, and phytotechnical procedures were applied according to the agricultural cultivation standard procedures [1]. Once completed their biological cycle, F1 plants seeds were harvested for each plant for subsequent application.

In vitro anther culture of F2 plants resulting from cross-breeding between selected progenitor cultivars

F2 plant seeds were seeded into the planting beds and 30 days later transplanted to field conditions into furrows separated 40 cm, 30 cm distance between plants, and applying phytotechnical procedures as recommended. The most vigorous and phytosanitary-adequate state F2 plants resulting from cross-breeding, grown for 60-70 days, were selected and two or three panicles collected attending to having a 4-8 cm separation between the auricles of the last two leaves. The knots and pods of the leaves were preserved to protect the leaves from the contamination of the field pathogens.

Then, collected vegetal material was processed at the tissue culture laboratory. Panicles were disinfected in the outer side with 70 % ethanol solution and further preserved with the lower part immersed into water, and they were protected with a dark plastic bag at 8-10 ºC for 7-8 days. Afterwards, panicles were disinfected for 3 min with 1.5 % sodium hypochlorite and 0.5 % Tween 80 as dispersing agent, and washed four or five times with distilled water. They were further seeded *in vitro* as recommended [15].

Then, three liquid culture media were tested for callus induction: N6-1 [16], N6-m and NL [15], three

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replicates each, and 100 anthers were seeded for each replicate. Once seeded, flasks were kept in the dark for 30-60 days until microcallus formation.

The number of callus-forming anthers per flask were evaluated. Calluses 1-2 cm wide were transferred to flasks containing solid minimal saline medium supplemented with 1 mg/L naphtaleneacetic acid (NAA), 2 mg/L indoleacetic acid (IAA), 4 mg/L 6-furfurilaminopurin (kinetin) and 30 g/L sucrose and 7 g/L agar. The flasks were incubated at 26 ± 2 ºC and under a 16 h light/8 h dark photoperiod.

Green and albino plants regeneration was evaluated after 30 to 40 days.

Selection and evaluation of double haploids combining good agronomic characters and drought tolerance

Water stress conditions were simulated by irrigation during germination, subsequently limited to water passes every seven days just to guarantee soil moisture equivalent to field capacity. Such conditions were maintained until the grains were pasty. The other agricultural practices were implemented as recommended [1].

Upon harvesting, plant height (cm) was assessed to 10 plants randomly selected; their central spikelets were evaluated for the number of filled grains per spikelet. Additionally, 1000 grains weight (g) and 1-m lined-up grains weight were determined, spikelet number, and spikelets were weighed to determine yields (kg). Maturation cycle was established in days, and industrial yield was estimated as the percentage of filled grains after every milling of a 100-g sample, resistance to lodging, shattering and pest incidence.

Statistical analysis

A statistical completely randomized design was applied, with three replicates for callus formation and nine for regenerated plants. Data were transformed by the squared root plus three eighths and statistically processed with the aid of Statgraphics Plus version 5. Results were analyzed by a simple classification variance analysis, and means were compared by the Tukey's test for a 5 % error. Quantitative data were subjected to Principal component analysis and multiple correlation with Statgraphics Plus version 5.

Results and discussion

Hybridization between cultivars selected for drought tolerance and good agronomic performance

Among the crossing which were able to produce grains (Table 2), only 42 % of all the 24 combinations tested formed seeds. This made impossible to complete the genetic design proposed.

Cultivar Amistad´82 seemed to have better combinatorial capacity than INCA LP-10 to form grains in artificial crossings. This coincides with previous reports on using *in vitro* anther culture to generate lines resistant to piriculariosis, using cultivar Amistad´82 as progenitor [8]. In our work, crossings were effective only when this cultivar was used as female progenitor.

The cultivar Perla de Cuba was the only drought resistant progenitor generating grains, when used as female progenitor. Despite, a number of combinations were obtained which could provide variability enough as to generate recombinants favorable for the selection of new cultivars.

Table 2. Direct and reciprocal crossings among rice cultivars resistant to drought and cultivars showing good agronomic performance which were able to form grains

No.	Resistant male parental (female/male)	No.	Resistant female parental (female/male)
1	Amistad'82/C4 153	13	NS
2	Amistad'82/IR 20	14	NS
3	Amistad'82/IR 36	15	NS
4	Amistad'82/Perla de Cuba	16	Perla de Cuba/Amistad'82
5	INCA LP 10/C4 153	17	NS
6	NS	18	IR1529-430/Amistad'82
7	NS	19	IR 1529-430/INCA LP 9
8	NS	20	Moroberekan/Amistad'82

NS: Reciprocal crossbreeding that did not generate seeds.

In vitro anther culture of F2 plants

A differential response was detected among the different hybrid combinations attending to callus formation per 100 anthers seeded (Table 3, Figure 1A and 1B). Not all the hybrids were able to form calluses, and variation was seen among those successful. The frequency of callus-forming anthers oscillated in the range 0-56.93 %, probably due to differences in genotype and culture media. Similar results were reported by different research groups while studying the influence of different culture media in calluses formation [8-11, 15], which could be attributed to heterozygosity in the loci controlling callogenesis.

The highest values were achieved in descending order by the INCA LP-10/C4 153 hybrid line in the NL medium (56.93 calluses per 100 anthers seeded), followed by this hybrid but in the N6-1 medium (34.80 %) and the hybrid Amistad´82/Perla de Cuba in the NL medium (45.06 %). A similar performance for the other two culture media, but at lower values. All these crossings included the Amistad´82 cultivar as female progenitor. By the contrary, other crossings including this cultivar either as male or female progenitor showed very low values (0-0.6 %), including Amistad´82/IR-20, Amistad´82/C4 153, Perla de Cuba/Amistad´82, IR 1520-430/Amistad´82, INCA LP-10/Amistad´82 and Moroberekan/Amistad´82.

Table 3. Callus formation per 100 anthers planted in three culture media, for F2 hybrid plants resulting from crossings between cultivars resistant to drought and cultivars of good agronomic performance*

Hybrid combination (female/male)	Calluses			Plants	
	NL	N6m	N6m	Green	Albino
INCA LP 10/C4 153	56.93 a	8.23 h	34.80 d	25.36 a	0 h
Perla de Cuba/Amistad'82	39.10 c	5.12 jk	20.10 f	20.16 b	10.56 b
Amistad'82/Perla de Cuba	45.06 b	6.50 i	28.23 e	14.40 c	7.33 c
Amistad'82/IR 20	0 p	2.30 l	0.26 p	0 h	0.90 fgh
Amistad'82/C4153	3.50 m	2.16 l	0.90 o	0.46 gh	3.26 d
Moroberekan/Amistad'82	4.70 l	0 p	5.30 j	8.16 d	1.56 ef
INCA LP-10/Amistad'82	8.46 h	2.23 l	4.76 kl	5.35 e	1.16 fgh
IR 1529/INCA LP-9	1.20 o	0 p	6.08 i	3.26 ef	0.46 fgh
Amistad-82/IR-36	0 p	0 p	0.06 p	1.70 fgh	2.63 de
IR1529/Amistad'82	16.20 g	5.30 j	0.20 p	0.33 gh	16.96 a
M	17.51	3.18	10.06	7.91	8.96
EEM	0.08	0.08	0.08	0.44	0.22

* Means sharing the same letter per column did not differ statistically, according to Duncan's test for 5 % error.

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Noteworthy, the INCA LP-10 cultivar generating the hybrid INCA LP-10/C4 153 is a somaclone of cultivar Amistad´82. Previous research on other crops like tobacco, tomato, potato, barley, soybean, wheat, flax and others, have found that genotype and culture media influence on diploid generation by *in vitro* anther culture [9, 10, 15]. In this sense, callus formation varied among the three culture media tested, with the highest values shown in the NL. The three media differ in salt concentration (macro and micronutrients), vitamins and growth regulators, thereby making difficult to correlate the observed effect of each culture medium to a single component. Nevertheless, the NL medium is the only one containing maltose instead of sucrose and containing silver nitrate. Other studies have shown that callus induction from recalcitrant genotypes and the production of green plants are enhanced by substituting sucrose by maltose. In fact, this change has been recommended for callus induction, particularly from the indica subspecies [8, 16].

On the other hand, the presence of silver nitrate diminishes anther senescence in recalcitrant indica rice. This result points towards the possible inhibition of callus formation by ethylene accumulated in the flask, an effect probably reverted by the application of the compound [16, 17].

Additionally, differences were found in the number of green and albino regenerated plants (Figure 1 C and D). The line INCA LP-10/C4 153 only formed green plants (25.36 plants per 100 anthers seeded), followed by the crossings Amistad´82/Perla de Cuba and Perla de Cuba/Amistad´82. Nevertheless, the IR 1529-430/Amistad´82 crossing showed the highest values in the formation of albino plants. The close genetic relationship between INCA LP-10 and Amistad´82 could explain the response seen. In this sense, variety C4 153 belongs to the japonica subspecies, which displays good performance in *in vitro* culture, specifically for anther culture [12, 16].

Regarding the formation of albino plants (Table 3 and Figure 1 D), the INCA LP-10/C4 153 was the only crossing not forming albino plants. IR 1529-430/Amistad´82, followed by Perla de Cuba/Amistad´82, showed the higher numbers, coinciding with the Amistad´82 as parental cultivar. Previous reports indicated that albino plants (i.e., chlorophyll deficient plants) are frequent among cereals obtained by anther culture techniques. In rice, the frequency of albino plants varies with the genotype, and a high rate of regenerants can be seen in certain cases, but the frequency can range 10-100 % [17]. Similarly, this phenomenon is particularly predominant among plants resulting from immature pollen of inter- or intra-specific hybrids between japonica and indica subspecies [15, 16]. Highly variable results have been reported, for instance, 57 to 75 green plants per 100 calluses and only 1-8 calluses per 100 anthers [18]; green plants were regenerated 15-30 days after culture with 33 % as the highest value obtained, with only 3-9 calluses per 100 anther and 1-6 plants per 100 calluses [19]. Differences not only among species attending to anthers but also among cultivars of the same species have been described [15, 18-20], with emphasis in the significant effect of the genotype on the regeneration of green plants.

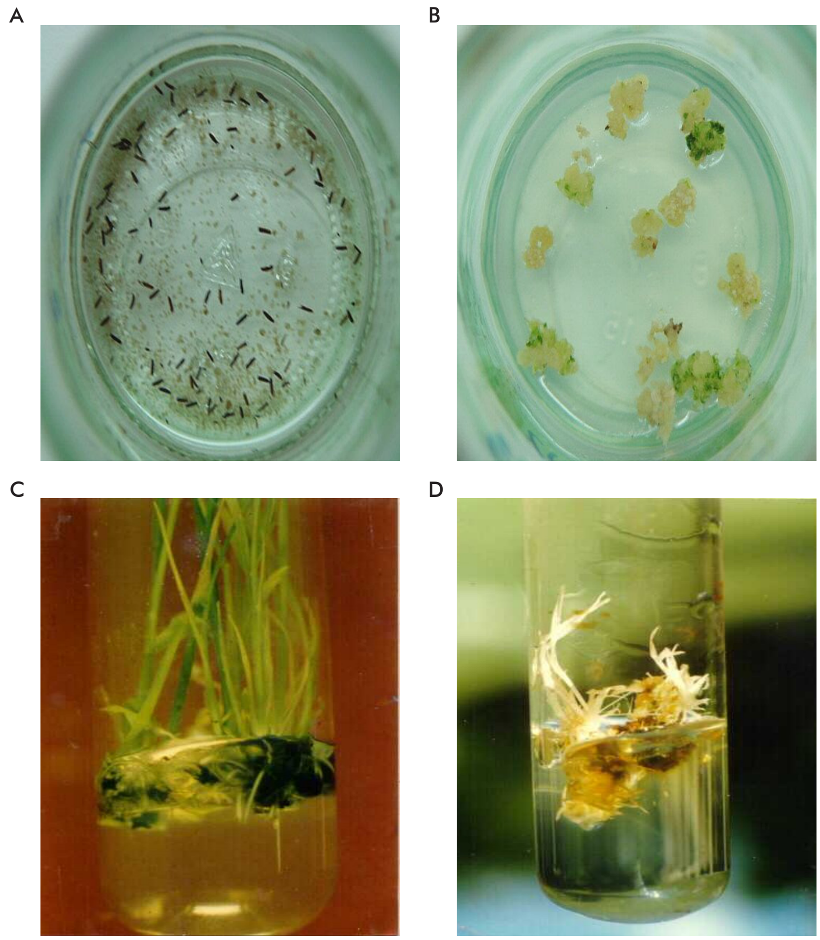

Figure 1. *In vitro* cultures of hybrid rice cultivars resistant to drought with cutivars of good agronomic performance. A) Anthers seedings in culture media. B) Callus formation. C) Green plant formation. D) Albino plant formation.

In general, it could be mentioned that the best results of callus formation from anthers of F2 plants were obtained with the NL culture medium and the hybrid combinations: INCA LP-10/C4 153, Amistad´82/Perla de Cuba and Perla de Cuba/Amistad'82. Coincidently, these were the three hybrids also showing the highest frequency of green plants in descending order. Otherwise, the IR1529/Amistad'82 hybrid showed the highest frequency of albino plants.

Selection and evaluation of double haploids integrating drought resistance and good agronomic performance

Seeds of plantlets obtained from regenerants of the different surviving hybrid combinations were harvested and planted under field conditions, and their morphological traits characterized and visually related to ploidy levels (Table 4). Haploid plants (n = x) are generally small, weak, stumpy and sterile. On the contrary, diploid plants exhibit favorable traits, they grow vigorous, fertile, with good development, and the polyploid plants further grow with exuberant flowering structures, a large number of grains with long spikelets and partial sterile grains [8, 15, 19].

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Considering the Principal components analysis run with the lines displaying phenotypic traits characteristic of diploid plants (Table 5), it was found that the two principal components C1 and C2 accounted for 90.89 % of the total variation, with C1 providing 10 % of the total variance explained (Table 5). The variable contributing the most to the C1 component was agricultural yield, and in C2 contributed with up to 90 % of the total variation. The variable 'filled grains per panicle' was the major one in a negative sense, and 'number of empty grains per panicle' in a positive sense. This could indicate the usefulness of these variable for differentiating among regenerants, due to their higher correlation values with the main axes.

Once represented the principal components C1 and C2 for genotype distribution, which were mainly influenced by the main evaluated variables (Figure 2), there was evidenced the existence of a high genetic variability among individual plants. This could derive from the diversity of the genetic material of the progenitors used for the crossing, since they have been obtained by different genetic improvement methodologies, each contributing a differential source of genetic variation. For instance, conventional methods, biotechnological techniques or their combination contribute different ranges of genetic variability. Moreover, parental cultivars resulted from genetic improvement programs for obtaining rice cultivar showing high yield potential and tolerant to different stress conditions, such as: salinity, drought and water-deficiency [12, 13].

Regarding the PCA analysis, the taller plant genotypes exhibiting the highest grain width and length and the longest panicles located in the C1 axis positive quadrant (Figure 2). Similarly, for the C2 axis, the best genotypes showing the best tolerance to water-stress, highest yields and higher number of filled grains per panicle were located in the positive quadrant, the most relevant example for the INCA LP 10/C4153 hybrid.

Other studies demonstrated that prolonged drought during the reproductive phase, which demands increased transpiration intensity, could lead to yield losses up to 85% despite high regenerant yields. Importantly, yield is a key character to choose which varieties will be transferred to field production. Hence, yield is established as depending on the components: panicle number, spikelet number per panicle, spikelet filling percentage and 1,000-grain weight. Nevertheless, there are situations in which the spikelet filling percentage becomes more restrictive for yields than spikelet number per panicle under certain climatic conditions. Therefore, the specific causes for yield variations need to be examined, as well as their components [1, 2, 12, 20].

Moreover, yield inheritance has been demonstrated low and a far complex parameter, since it is influenced not only by the genes involved in defining the yield components, but also other characters indirectly related to yield. Among these last are plant height, the cultivation cycle and the grain-associated characters. Noteworthy, the environmental influence on character expression should be taken into account [20].

Conclusions

The new genotype established was determinant for the successful *in vitro* anthers culture in F2 plants. Moreover, the cultivars' combinations INCA LP-10/ C4 153, Amistad-82/Perla de Cuba and Perla de Cuba/ Amistad-82 showed the best responses of callus formation and regeneration of green plants. The NL culture medium provided the best among the three media tested for callus formation from F2 plants

Table 4. Regenerants evaluated per crossing, displaying diploid-like morphology

No.	Resistant male parental (female/male)	Evaluated lines
1	INCA LP 10/C4 153	15
2	Perla de Cuba/Amistad'82	10
3	Amistad'82/Perla de Cuba	6
4	Amistad'82/ C4 153	2
5	Moroberekan/Amistad'82	4
6	INCA LP-10/Amistad'82	2
7	IR 1529-430/INCA LP-9	1
8	Amistad'82/IR-36	1
9	IR 1529-430/Amistad'82	1
	Total regenerated plants	42

Table 5. Eigen values, contribution percentage and total of components 1 and 2 and their correlations with principal components

Parameter	Principal components	
	C1	C2
Eigen value	80.873	80.873
Contribution (%)	10.018	90.871
Cumulative (%)	–	90.891
Height	0.0056	0.0447
Panicles/m^3	0.1529	0.0267
Panicle length	0.0035	0.0022
1,000 grain weight	–0.0009	0.0048
Yield	0.9866	0.0602
Filled grains/panicle	0.0450	–0.7259
Empty grains	–0.0454	0.6830
Grain length	0.0017	0.0063
Grain width	0.0022	–0.0015

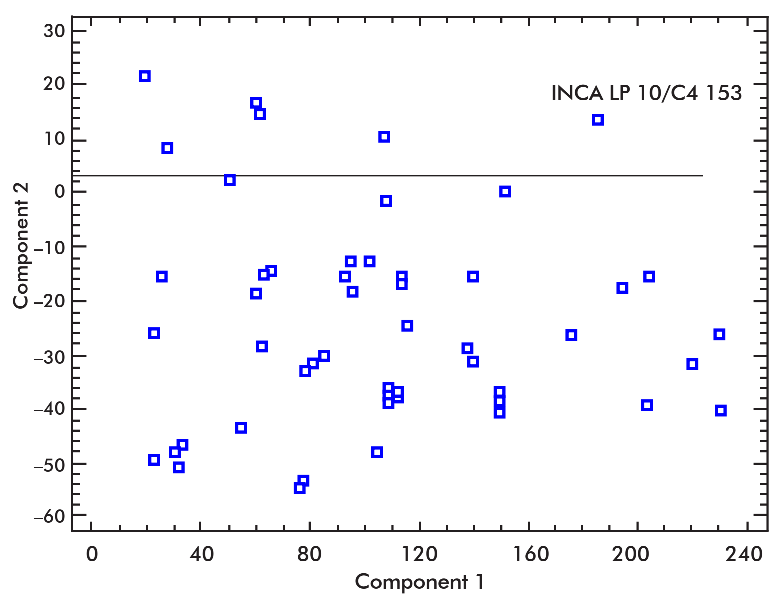

Figure 2. Distribution of regenerants according to the two principal components in PCA analysis for the variables studied.

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anthers. Advantageously, the method introduced shortened the lines' improvement cycle as compared to traditional cross-breeding techniques, with the expected variability and character fixation through *in vitro* F2 plants anther culture. It was also possible to detect the regenerant INCA LP-10/C4 153 in the third generation under water stress conditions, which provided high yields and the highest panicle number. Significantly, a high genetic variability was found among all the regenerants tested.

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Conflicts of interest statement

The authors declare that there are no conflicts of interest.