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Rice cv. Bahia mutagenized population: a new resource for rice breeding in the Mediterranean basin

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Abstract

In the Mediterranean basin, the limited availability of rice genetic resources restricts the success of breeding programs. Although mutation induction is a powerful alternative to generate desired novel variations, current rice mutant resources have little relevance in the Mediterranean breeding programs that require well-adapted cultivars for maximum performance. In this work a genome-wide mutation induction has been used on rice cv Bahia, a *japonica* cultivar well adapted to the Mediterranean climate and cultural practices, to increase its genetic variability and to facilitate the generation and selection of interesting agronomical traits. Bahia mutant collections were generated by irradiating seed batches with either 30 Gy fast neutrons or 250 Gy gamma rays. After initial screening of M2 plants and followed by evaluation of M3 and M4 generations in open field, elite genotypes were selected. The selected lines carried morphological and physiological changes of agronomical interest such as earliness, culm height reduction, yield increase and spontaneous spots appearance in leaves. The mutants are useful as breeding materials for improving varieties in the Mediterranean.

Additional key words: fast neutrons, gamma rays, genetic variability, mutant, *Oryza sativa*.

Resumen

Una colección de mutantes de arroz de la variedad Bahía: una nueva herramienta para la mejora de arroz en el área Mediterránea

En la cuenca mediterránea, la escasa disponibilidad de recursos genéticos de arroz es un factor limitante para los programas de mejora. A pesar de que la inducción de mutaciones es una alternativa poderosa para generar variaciones genéticas, los recursos ya existentes de mutantes de arroz son de escasa utilidad para los programas de mejora en el Mediterráneo, ya que requieren variedades bien adaptadas al clima para obtener una máxima eficiencia. En este trabajo, se han generado mutaciones genómicas al azar en la variedad *japonica* Bahía, bien adaptada al clima y las prácticas agrícolas mediterráneas, para aumentar la variabilidad genética y facilitar la generación y selección de innovaciones en caracteres de interés agronómico. La colección de mutantes de Bahía fue generada irradiando grupos de semillas bien con neutrones rápidos, 30 Gy, o bien con rayos gamma, 250 Gy. Tras rastrear plantas M2 y caracterizar las subsiguientes M3 y M4 en campo, se seleccionaron fenotipos élite. Las líneas mutantes seleccionadas presentan cambios morfológicos o fisiológicos como acortamiento del ciclo vegetativo, reducción de altura, incremento en producción o desarrollo espontáneo de manchas necróticas en hojas. Los mutantes obtenidos tienen utilidad como material de partida en programas de mejora de variedades en la zona mediterránea.

Palabras clave adicionales: mutante, neutrones rápidos, *Oryza sativa,* rayos gamma, variabilidad genética.

Introduction

Mutagenesis is a useful technique to increase genetic variability in plant populations. Since 1960s, several collections of mutant lines from different species have been isolated and successfully used in many different areas of plant biology and crop breeding. New varieties have been developed either directly after mutagenic treatment or through crosses involving mutant varieties or mutant lines. In 2000, the IAEA (International Atomic Energy Agency) recorded 2,252 different mutant cultivars that had been officially released. Out of these new cultivars, 1,585 were generated directly after mutagenic treatment and selection in subsequent generations (Maluszynski *et al.,* 2000). This technique has had great impact on the income of farmers and national economies, for example, in China and Japan. Among the 1,585 new

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cultivars, 434 were derived from rice mutants generated with different mutagenic agents, largely gamma radiation (no less than 166). Semi-dwarfism and earliness are the characters most frequently described in released rice mutant cultivars, although other desired traits such higher stem number, improved grain quality, blast tolerance, photoperiod insensitivity and salt tolerance are also common (Maluszynski *et al.,* 1995). There are many examples on the development of new and valuable alterations in rice characters and on their contributions to improve specific cultivars with economic impact. For instance, cv. Zhefu 802, which shows earliness and high yield potential as improved traits, was grown in more than 10.5×10^6 ha at the beginning of 1990s in China (Liu *et al.,* 2004).

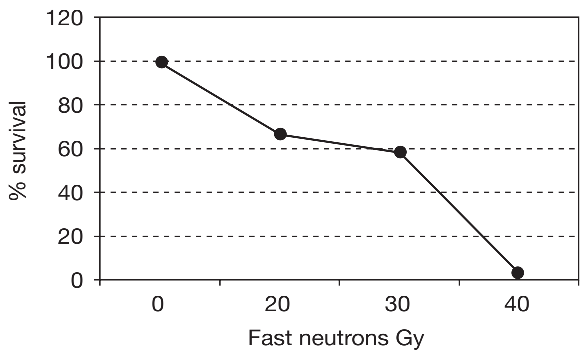
Chemical and radiation mutagenesis can produce a large number of functional variations in rice, although the efficiency of the several mutagenic agents may vary (Rutger, 1992). Gamma rays (GR) are the most popular mutagen followed by others such as X rays and fast neutrons (FN). Radiation dosage and mutation frequency depend on the type of mutagen and species. In rice, to allow 60% survival (percentage of seeds germinated and developed to adult plants), effective dosage of GR generally ranged from 150 to 300 Gy and from 18 to 35 Gy if FN are used (Rutger, 1992; Li *et al.,* 2001; Wu *et al.,* 2005). Calculations performed in Arabidopsis indicated that 2,500 lines treated with FN at 60 Gy are required to inactivate a given gene (Koornneef *et al.,* 1982), assuming that 10 genes are randomly affected in each line (Li *et al.,* 2001). Similar mutation frequencies have been observed in plant species of different genome sizes (Koornneef *et al.,* 1982).

Changes produced by irradiation include chromosomal alterations (deletions, translocations, inversions) and point mutations. Reported examples of mutants generated by FN revealed several kilobase deletions (Sun *et al.,* 1992). Radiation produced by X rays and GR are less energetic than FN, mostly induced single or few base pair changes, however, examples of varying deletion were documented, ranging from few pair of bases deletions or insertions to larger deletions affecting several genes (Yano *et al.,* 2000; Yamanouchi *et al.,* 2002; Haga *et al.,* 2004). Experiments performed at IRRI (International Rice Research Institute, www.iris.irri.org) to detect genomic changes in the IR64 mutant collection generated by either GR or FN, indicated that many deletions were greater than 1 kb (Wu *et al.,* 2005).

Current breeding goals in rice, a major staple food source worldwide, are to improve production efficiency and to maintain high quality and yield. Decades of traditional breeding, however, have led to the replacement of many local cultivars reducing genetic diversity, a factor limiting the introduction of more new traits. Major traits that need improvement are yield, grain quality (e.g. shape and amylose content), nutritional and cooking grain quality (e.g. protein content and consistency), insect and fungal resistance, and abiotic stress tolerance (e.g. drought and wind). The need for wide genetic variability in parental lines have frequently forced the use of foreign germplasm carrying many characteristics that could spawn offspring unacceptable for production or marketing in the needed region (e.g., cooking quality, time of maturing, milling quality and grain shape). As explained above, mutation induction has been used as a powerful alternative to generate desired novel genetic sources for particular plant characters. Currently, rice mutant resources include collections that were generated by insertional, radiation or chemical mutagenesis (Hirochika *et al.,* 2004; Kurata *et al.,* 2005), but only irradiated or chemically induced rice mutants have been proved to be very useful in plant breeding programs, providing excellent varieties in a direct or indirect way. However, there are only few public available mutant collections based on induced mutations, including an *indica* collection of 50,000 M3 and M4 mutants generated from IR64 at IRRI and a *japonica* collection of 6,000 mutants derived from Kinmanze and Taichung 65 at Kyushu University (Inst. of Genetic Resources, www.shigen.nig.ac.jp/rice/oryzabase). Unfortunately, the plant materials of these two robust collections are of limited relevance in the Mediterranean breeding programs because the dramatic influence of local climate and cultural practices on rice cultivar development. The objective of this work is to generate, using GR and FN, a new mutant collection from *japonica* Bahia that is well adapted to the Mediterranean climate and carries many desirable agronomic characteristics. The mutant collection will allow the identification, isolation and propagation of mutants with improved morphological and physiological traits at vegetative and reproductive stages to be used in breeding programs in the Mediterranean basin.

Material and Methods

Seeds from *Oryza sativa* cv Bahia (*japonica*) were used in the irradiation experiments. To optimize the



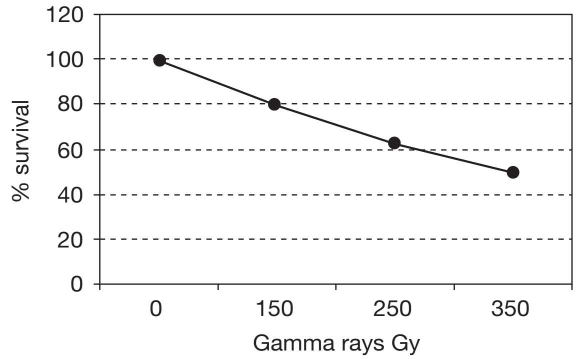


Figure 1. Response to different dosages of two mutagenic radiations. Effect of different dosages of fast neutron and gamma rays radiation on survival of plants in rice cv. Bahia. The percentage of growing plants was scored 21 days after germination.

dosage of irradiation, preliminary assays were performed by irradiating two sets of 100 seeds each with either FN at 20, 30 and 40 Gy or with GR (^{60}Co) at 150, 250 and 350 Gy. Based on the results in these pilot experiments, successive batches of 2,500 seeds were mutagenized with FN at 30 Gy at the *Instituto Tecnologico e Nuclear* (Sacavem, Portugal) and with GR at 250 Gy at the *Hospital Provincial de Castellón*. The irradiated seeds were germinated in nurseries and then transferred to open fields. Plants grew during summer months, flowered in August and reached maturation at the end of September. At maturity, every five M1 plants that were fertile were grouped into a family and M2 seeds from each family were bulked. A total of 125 seeds per M2 family were sown in nurseries and after five weeks seedlings were transplanted manually to soil in open fields. Plants were grown during summer and first screening was performed during the late vegetative and reproductive stages for characters of culm height, heading date, presence of necrotic lesions, grain size and shape, and lodging resistance. The frequency of appearance of the phenotypic categories was measured. During two consecutive years, 18,700 M2 GR plants and 8,300 M2 FN plants were screened. Each M2 plant was considered to be an independent line. Seeds from selected M2 plants were harvested and sown again. Ten M3 plants derived from each of the selected M2 plants were grown during winter in the greenhouse at 22ºC. The phenotypes of the mutants were further confirmed and M3 plants were selected. During the following summer, 75 M4 seeds from each of the selected M3 plants were sown in separate rows in fields (15 × 40 cm) to produce enough seeds for storage and further experiments. Non-radiated Bahia plants were grown as control and Montsianell cultivar was used as height control. The following characters were evaluated for plants in M3 and M4: height, heading date, number of fertile panicles per plant, number of grains per panicle and 1,000 grain weight.

Results and Discussion

Mutagenesis

Pilot experiments were carried out on Bahia cultivar to determine the optimal dosage of FN and GR. The results revealed that the doses to allow 60% survival in Bahia cultivar were close to 30 Gy FN and 250 Gy GR (Fig. 1). The severity of the mutagenesis was indicated by the number of M1 plants that did not produce seeds and the number of albino plants present in the M2 generation (Table 1).

Thereafter, two separate sets of 2,500 Bahia seeds each were irradiated with either 250 Gy GR or 30 Gy FN. Most of the M1 plants showed retarded growth, low density panicles and high sterility due to the muta-

Table 1. Frequency of some phenotypic mutations among M2 Bahia plants. Values are the percentage of plants among mutants produced with each mutagenic agent. Each trait is based on observation of 18,700 M2 GR plants and 8,300 M2 FN irradiated plants

	Fast neutron mutagenized lines	Gamma ray mutagenized lines	Total mutagenized lines
Early heading	0.19	0.15	0.17
Short culm[a]	0.51	0.60	0.57
Spotted leaves	0.25	0.16	0.19
Albinos	0.54	0.59	0.56

[a] Short culm includes dwarf and semi-dwaf plants (shorter than 85 cm).

genic effects, while about 1,200 plants in each treatment were fertile. During two consecutive years, M2 plants were grown in open fields and a first screening was performed during the late vegetative and reproductive stages for variations in characters.

M2 plant screening

A wide range of visible morphological changes were observed among M2 plants: earliness, development of spontaneous lesion spots, culm height, leaf shape and color, vigor, number of tillers, grain type and yield. Several plants showed concurrent or combined characters and, frequently, more than one plant from the same family showed the same type of variation, indicating a change of genetic nature. M2 plants were screened for early flowering, necrotic lesion development and reduced culm length and the frequency of these changes was detailed in Table 1. A total of 285 plants were selected as putative mutants of interest. Further analyses of the phenotypes of M3 plants grown in both greenhouse and open field allowed the characterization of the new mutant lines.

Early flowering mutants

Reduction of rice vegetative growth duration offers farmers several advantages such as diminution of the damage risk caused by storms or other natural disasters and broadening in harvesting time. Furthermore, in template climates a considerable reduction of the vegetative growth period may allow two annual harvests, as observed in the long grain cultivar Labelle, a mutant cultivar introduced in USA that can produce a second yield if the south areas where season is long enough.

Several early mutants were found in the screening. The average plant height and tiller number of these early flowering mutants were in general reduced and in consequence they were low yielding lines. For instance, plants from mutant lines S41.01 and T03.01 are two weeks earlier than Bahia at maturity. Consequently, these mutant plants are shorter than Bahia (Table 2) and also produced fewer tillers. Moreover, line S73.04 has very shorter life cycle and photoperiod insensitivity. These plants came to heading 6-7 weeks after sowing both in open field while Bahia plants came to heading 9-10 weeks. In greenhouse, independently upon the photoperiod exposure, line S73.04 showed the same heading date as in open field. The plants of line S73.04 also become pale yellow two weeks after germination and the yellowing persisted until maturity. The adult plants of line S73.04 are weak and have fewer tillers and grains per plant despite normal shape of grain.

Table 2. Height of relevant mutant lines at maturity. Bahia and Montsianell, two wide grown cultivars in the Mediterranean area, were used as controls

Line	Heigth (cm)
T27.07	58.6 ± 2.8
T100.05	63.8 ± 2.0
S65.03	75.2 ± 3.2
S73.04	76.0 ± 5.2
T03.01	78.1 ± 3.4
S65.01	79.1 ± 2.9
S41.01	80.9 ± 3.4
T67.03	84.0 ± 5.8
S73.07	87.4 ± 2.4
S29.01	92.0 ± 3.0
S70.07	98.2 ± 3.3
Montsianell	100.7 ± 3.4
Bahía	119.5 ± 2.0

Lesion mimic mutants

Plants respond to pathogenic attack through the activation of defense mechanism including the hypersensitive response (HR). In HR, cell death is induced in the infected areas in order to isolate the pathogen to avoid its spread out to the rest of the plant. HR may also be accompanied by the synthesis of reactive oxygen species (ROS), the activation of specific defense genes, the accumulation of antimicrobial compounds and cell wall alterations. Moreover, HR may involved systemic acquired resistance (SAR) conferring resistance to successive pathogenic attacks.

Natural or induced mutants that show activation of HR in the absence of pathogens have been described in numerous species. These mutants developed spontaneously necrotic spots and, for this reason, they were called disease lesion mimic (LM) mutants. In many of instances, the lesions were induced in response to non pathogen related stimuli like alterations in physiology or environmental conditions such as high temperature and solar radiation (Yamanouchi *et al.,* 2002). LM mutants are usually plants with the defense system activated constitutively and lesions occasionally appear coupling with the activation of defense genes and SAR. The defense response triggered in these mutants can be unspecific for a wide range of pathogens.

In the initial screening for LM mutants in Bahia M2 lines, several plants that developed spontaneously necrotic lesions were identified. The lesion mimic phenotype was confirmed in at least 10 lines in the M3 and M4 generation. All these mutant lines were normal in growth and showed green leaves and healthy grains, except for the development of necrotic lesions. Interestingly, not all lines developed lesions when grown in the greenhouse although all of them showed this phenotype in open field. This observation is not surprising since the appearance of necrotic lesions depends on physiological and/or abiotic stress signals (humidity, sunlight, mechanical aggression, winds, etc.) and that may not exist under controlled environment. It is worth to note that these plants that did not produce necrotic lesions in the greenhouse showed in contrast a very healthy aspect and kept the natural green color longer than wild type. In general, the appearance of the necrotic spots varied in every Bahia mutant, although most of them consisted of long brown areas with different shapes and length (Fig. 2). The time of appearance of the spot during plant growth was also different for each mutant, e.g. line B11.01, developed spots after eight weeks whereas T67.03 did after 5 weeks of growth.

Short culm mutants

Plant height is a fundamental trait for breeding. In rice, higher yields are usually obtained from shorter crops because the reduction of stature increases lodging resistance. Among the whole range of rice cultivars with different height, semi-dwarf ones are the highest yielding since a severe reduction in height tends to decrease production and to hinder development during early stages of growth and also at harvesting.

Reduced height was the most frequent characteristic found among Bahia mutants. The selected M2 plants that showed reduced height were grouped in 63 independent mutagenic events. According to the final height that the mutants reached, they were classified by comparison with Bahia wild type (120 cm) into three clusters: shorts (86-100 cm), semi-dwarfs (70-85) and dwarfs (< 70 cm). Among the dwarf mutant lines found, some might be useful for breeding since they were normal in growth rate. Other lines showed grassy aspect with high number of tillers and lack of seeds.

The plant heights were compared between several M3 lines and cultivars Bahia and Montsianell that are widely grown in the area (Table 2). Several of these M3 lines were further characterized. For instance, S65.03 in M3 and M4 generations showed short stature in both open field and greenhouse conditions (Fig. 3). The reduction of growth in this line that reached a final height of 75 cm was evident at very early stages of growth. The mutant had short internodes with green and fully expanded leaves and small grains. Another interesting line, S65.04, came from the same M1 family of line S65.03, had shorter stems (60 cm) and normal grains (Fig. 4). Similarly, line S50.05 was 73 cm tall and developed grains of normal shape and size (Fig. 3) whereas line T100.04 was 75 cm tall and developed dark green leaves and grains.

Other characteristics

The mutation screening also revealed the presence of several lines with variations in different traits. Although both lines B03.03 and S77.02 had shorter panicles (Fig. 5), the latter also had small grains lacking the white core from Bahia (Fig. 4). Line S75.01 plants, for example, had a very robust appearance and their erect leaves and panicles did not bend at any time. In addition, line S75.01 plants also had shorter stature, slightly smaller and round grains (Fig. 4) and dark green leaves. Line T01.03 had one single and shorter stem, erect leaf close to 90° angle and one week earlier heading date. Other mutant lines that showed large variations

Table 3. Results of preliminary yield of mutant lines S12.01, S70.03 and S71.01. Plants were grown in rows in open field at a separating distance of 15 × 40 cm

Line	Height (cm)	No. fertile panicle per plant	Panicle weight (g)	1,000 grain weight (g)	Plant grain weight (g)	No. grains per plant
S71.01	108.1 ± 5.5	22.4 ± 6.6	4.4 ± 0.7	32.9 ± 2.0	87.6 ± 18.9	2,694.8 ± 647.6
S70.03	98.1 ± 3.1	28.5 ± 6.1	3.2 ± 0.5	34.2 ± 1.2	89.5 ± 15.2	2,314.3 ± 448.8
S12.01	119.0 ± 2.1	25.3 ± 6.5	5.0 ± 1.0	34.6 ± 1.2	103.9 ± 20.7	3,004.2 ± 629.2
Bahia	119.5 ± 2.0	17.0 ± 3.2	4.1 ± 0.7	37.0 ± 0.7	63.9 ± 9.5	1,724.7 ± 250.7

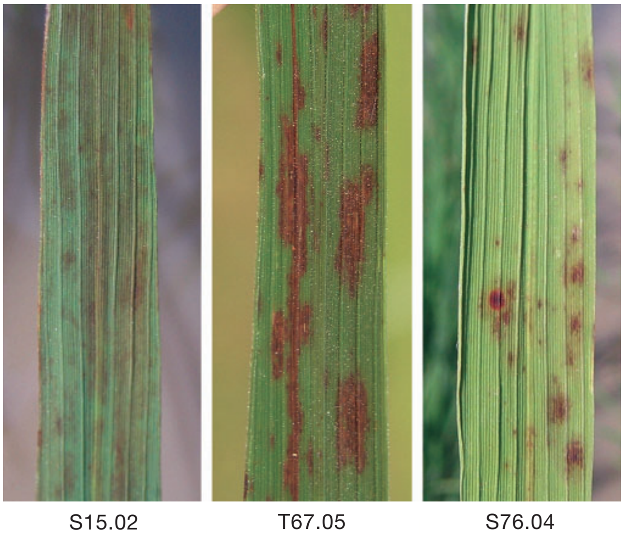


Figure 2. Leaves from three different mutant lines displaying necrotic lesions.

in other traits were also recorded. Line B09.03 developed purple spots in the sheath and lamina of the leaf in the area next to the collar (Fig. 6). Several lines produced higher yield as well as number of grains due to higher number of panicles and/or more grains per panicle. The phenotype of three of such lines was confirmed in the M4 generation grown in both field and controlled conditions (Table 3). In open field (15 × 40 cm), line S71.01 produced, in summer 2005, 22.4 panicles per plant with an average of 4.4 g per panicle while in the same conditions Bahia developed 17 panicles with 4.1 g per panicle. This line was also slightly shorter than Bahia (Table 3). The other two lines S70.03 and S12.01 had similar yield and number of grains to line S71.01 (Table 3).

In conclusion, a Bahia mutant collection has been generated intending to be a public resource for rice breeding programs. Since Bahia is a well-adapted cultivar to the Mediterranean climate these induced mutants are useful as genetic sources to improve and renew rice varieties in this region. Well-appreciated variations in agronomic traits have been found such as earliness, spontaneous lesion spots development, culm height, leaf shape and color, vigor, number of tillers, grain type and yield. In the future, new searches for phenotypes related to other important traits, such as drought tolerance and pathogen resistance, will be carried out in collaboration with other researcher groups.

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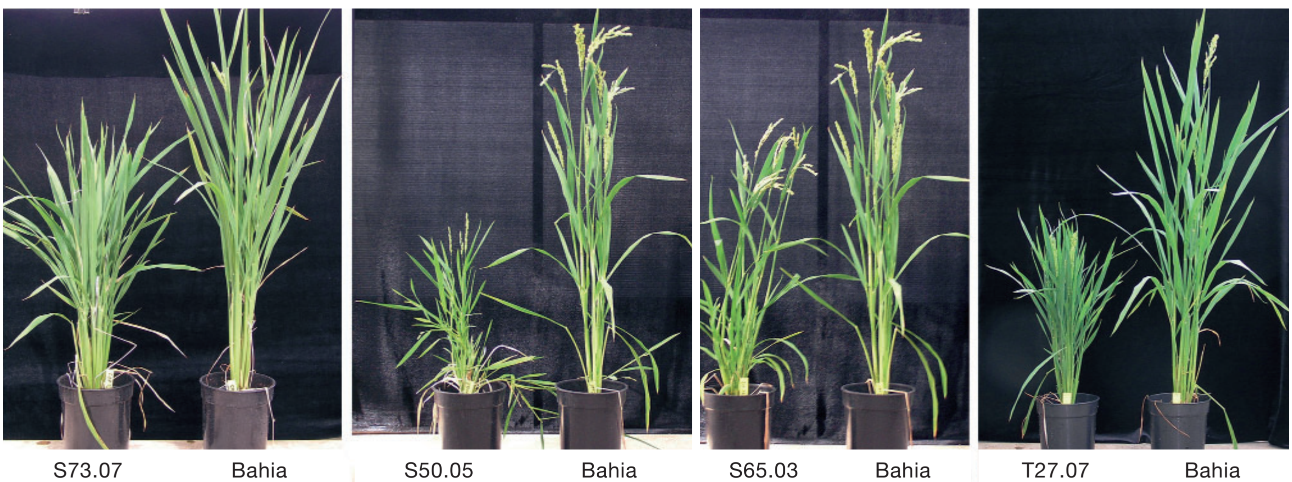

Figure 3. Mutant lines with shorter culm length. Plants from S50.05, S65.03, S73.07 and T27.07 lines are shown compared to Bahia control.

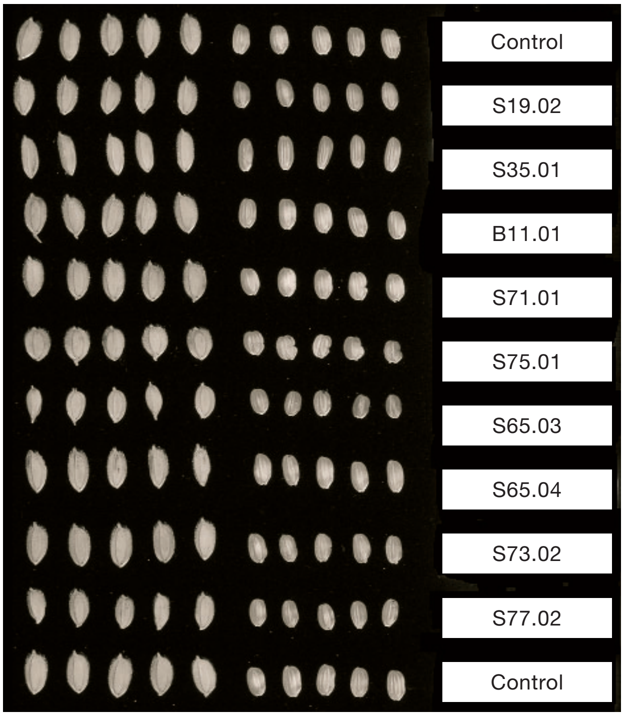

Figure 4. Variation in grain shape of some mutant lines compared to Bahia control.

Figure 6. Mutant line B09.03 development purple spots in leaf area next to the collar.

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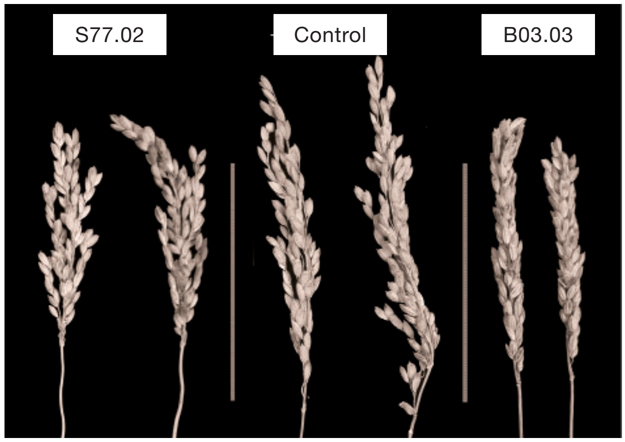

Figure 5. Mutant lines S77.02 and B03.03 had shorter panicles than Bahia control.

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