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Breeding Note

Alteration of resistance to black Sigatoka (*Mycosphaerella fijiensis* Morelet) in banana by *in vitro* irradiation using carbon ion-beam

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Abstract Carbon-ion beam is a new irradiation source for inducing mutations in plant breeding effectively. In order to apply this new technique in banana breeding program, we studied the critical doses for *in vitro* irradiation and the genetic variability for black Sigatoka in the regenerated plants. Carbon-ion beam was irradiated to *in vitro* plantlets of banana cultivars 'Cavendish Enano' and 'Williams' with the dose of 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 Gy. Biological effects on survival rate were recorded and 8 Gy was supposed to be the best dose. Survived plantlets were propagated *in vitro* to evaluate resistance to black Sigatoka. Six plants from 'Williams' population and two plants from 'Cavendish Enano' population were selected as candidates for resistant plants to black Sigatoka in the field, suggesting that carbon-ion beam could be useful for mutation breeding in banana.

Key words: Banana, black Sigatoka (*Mycosphaerella fijiensis* Morelet), carbon ion-beam, juglone.

Banana (*Musa acuminata*, AAA) is an important crop extended in tropical and subtropical regions around the world. Low reproductive fertility and high polyploid levels of banana make the traditional hybridization breeding techniques remain difficult (Rowe 1984). Most of the researches aim at finding out tolerant/resistant cultivars to black Sigatoka (*Mycosphaerella fijiensis* Morelet). This disease is one of the most serious constrain for banana cultivation, being the most destructive disease which attacks the leaves (Craenen and Ortiz 1996).

Using gamma rays, Roux (2004) reported different banana mutants with improved morphological characteristics of bunch size and cylindrical shape (mutant line name: 'Klue Hom Thong KU1') in Thailand, and plant height (dwarfness) (mutant line names: 'SH-3436-L9' and '6.44') in Cuba. Mutants with increased tolerance to *Fusarium oxisporium* (mutant line names: 'Mutiara' and 'Novaria') in Malaysia and to toxin of *M. fijiensis* (mutant line manes: 'GN35-I to GN35-VIII') by IAEA were also reported.

The ion beam technique has been used recently to produce a wide range of mutants rather than gamma rays. Fukuda et al. (2004) mentioned that ion beams can frequently produce large DNA alteration such as inversion, translocation, and large deletion rather than point mutation, resulting in producing desirable characteristics. Yu (2006) have mentioned that the most important application of artificially induced mutations is direct to the mutation breeding, using sexual or asexual offspring to induce ideal genotypes. Since the biological effects of ion beam as a new mutagen were discovered, this technique has received progressively more attention. Ion beams integrates the factors of mass, energy and charge, inducing damage to the biological materials, thereby displacing, recombining and compounding the biological molecules and atoms.

The present study is the first report using ion-beam irradiation for mutation breeding in banana for selecting lines tolerant to black Sigatoka. The effect of irradiation doses on the regeneration of plantlets was investigated in Japan, and the variation of the black Sigatoka response under field condition was evaluated in Ecuador.

Four-week old *in vitro* propagated shoot tips from two cultivars of banana 'Williams' and 'Cavendish Enano' belonging to the Cavendish subgroup and highly

Abbreviations: DDP-days, Disease development period; II-%, infection index; LDNA-%, leaf disc necrotic area.
This article can be found at http://www.jspcmb.jp/

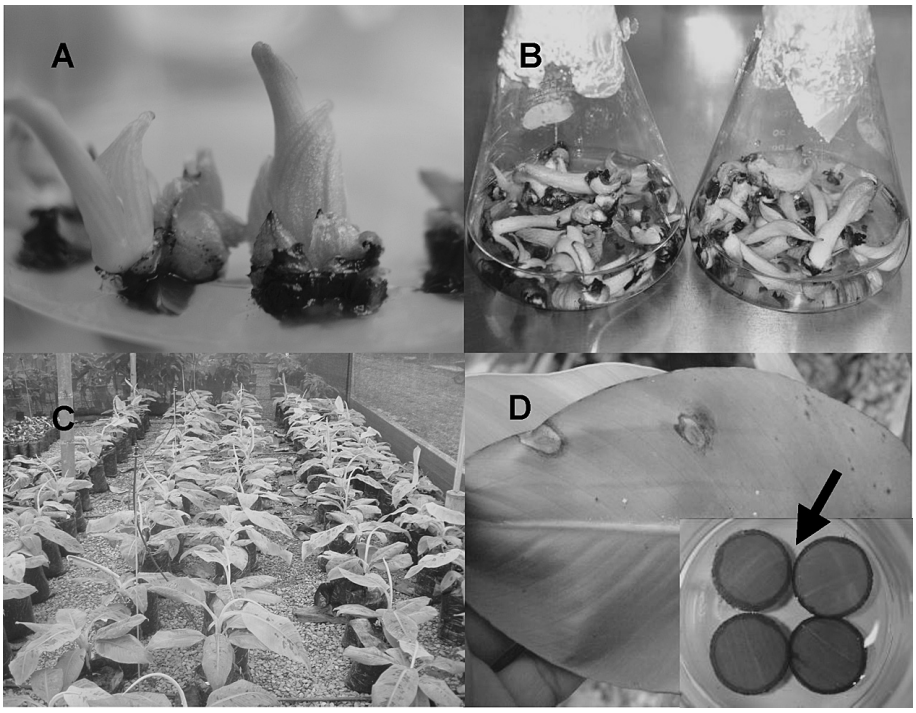

Figure 1. Plant regeneration and juglone toxin inoculation. Shoot tips (A), sub-cultured explants after irradiation (B), plants at nursery for inoculation (C) and young leaf affected by black Sigatoka (D). Arrow shows less affected leaf discs by juglone toxin.

susceptible to black Sigatoka were used. Solid MS medium (Murashige and Skoog 1962) supplemented with BA (2.25 mg l^{-1}), IAA (0.05 mg l^{-1}), sucrose (20 g l^{-1}) and agar (9 g l^{-1}) at pH 5.6 was used for *in vitro* culture. Shoot tips (Figure 1A) were sliced and placed in 6 cm Ø plastic dishes containing the solid MS medium for two days before irradiation. Twenty explants/dish×2 dishes (40 explants per dose) were used. The dishes were covered with sterilized Kapton films of 8 μm thickness (Toray-Dupont, Japan) to prevent the loss of energy of the carbon ions during irradiation.

The irradiation was conducted at the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA), Japan Atomic Energy Agency (JAEA). A total energy of 320 million electron volts (MeV) was generated by an AVF cyclotron. The physical properties are as follows: 311 MeV (25.9 MeV/u) as the incident energy at the target surface, 2.2 mm as the range of the ions in a target, and 137.6 keV/μm as the mean linear energy transfer (LET) in a target. The samples were irradiated with doses of 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 Gy, and maintained at tissue culture room conditions. Two days after the irradiation, the explants were transferred into a 100 ml Erlenmeyer flask containing 50 ml of liquid MS medium, and cultured on a rotary shaker at 100 rpm at 26°C in 16-h light/8-h dark (light intensity of 65 μmol m^{-2} s^{-1}). Survival rate (%) of explants was evaluated after 19 days.

Irradiated explants were sub-cultured three times to regenerate shoots (Figure 1B). Then these shoots were planted individually into a test tube containing 10 ml of solid MS medium, and 0.5 mg of activated carbon for rooting. Then the shoots were cultured in the same conditions above. Eventually, a total of 1,707 rooted plantlets were transferred to sterilized plastic bags and transported to Ecuador (Estación Experimental Tropical Pichilingue, INIAP) for field experiments.

Immediately after arrival in Ecuador, plantlets were kept for two days in the tissue culture room at 26°C under light conditions 16-h light/8-h dark (82 μmol m^{-2} s^{-1}), to recover photosynthesis, then transferred on a soil substrate bed (with 1 : 1 mixture of soil and decomposed rice husk) and covered with a plastic sheet to avoid dehydration under greenhouse conditions. Only 87 plants have survived after the nursery acclimatization and the high mortality might be caused by unfavorable transportation conditions. Using these materials, the experiments for black Sigatoka inoculation at nursery and later on, at the field conditions were performed.

Three banana leaves per plant were inoculated. Plants were about 30 cm in height (Figure 1C) and the younger expanded leaf was marked as the first leaf for inoculation by a conidial solution. Second and third successive young emitted leaves were inoculated by fragments of the diseased banana leaves. Conidial cultures of *M. fijiensis* in a concentration of 1.5×10^{6} ml^{-1} were obtained. After inoculation, the plants were kept at 26°C with a high relative humidity (approximately 85%) in a dark incubation room for 48 h. For second and third

leaves inoculation, leaf fragments completely diseased by black Sigatoka were placed at the base and inside canopy of each plantlet as a potential natural inoculum. A fickle cotton sheet moisturized thrice a day, was provided to cover all the plantlets to ensure the sporulation and enhance the inoculation.

Tolerance to black Sigatoka on inoculated banana plants was evaluated by three indices; DDP-days, II-% and LDNA-%. DDP-days was expressed as days until the full development of the spot with dry gray center (Figure 1D), using the stages of symptoms described by Fouré's scale from the inoculation time (Orjeda 1998). The disease severity determined by II-% was calculated using the values obtained from the Stover's scale modified by Gauhl (Orjeda 1998). For LDNA-%, five leaf discs of 15 mm-diameter per plant were prepared using a cork borer from the second expanded leaf. A solution containing 150 ppm of juglone (5-hydroxy-1, 4-naphthoquinone), a most active toxin produced by *M. fijiensis* (Strobel et al. 1993) was used for inoculation and the measurement of the LDNA-% was performed following the protocol by Reyes-Borja et al. (2005). A total of 435 leaf discs from both cultivars were analyzed (87 plants×5 leaf discs/plant). Data were processed by using frequency distribution and linear regression.

The survival rate (%) of irradiated explants is shown in Figure 2. There was no marked reduction on the survival rate up to ≦8 Gy. The initial growth of the plantlets was highly affected by higher doses than 8 Gy, and the leaf growth was very slow and it showed abnormal shapes, indicating that 8 Gy was the shoulder dose of *in vitro* carbon-ion beam irradiation in banana. Okamura et al. (2003) analyzed the mutagenic effect of carbon ions on carnation color and they showed that the widest variety of mutants was obtained at the shoulder dose of the survival curve and LD_{50}. From these facts, around 8 Gy was supposed to be the best dose for mutation induction in banana.

As shown in Figure 3A and D of the frequency distribution by class limits of the DDP-days in both cultivars, the higher class limits values were clearly separated from the lower as indicated between dotted vertical lines, ranging from 53.0 to 59.9 days and from 50.0 to 54.9 days in 'Cavendish Enano' and 'Williams', respectively. Lowest II-% values in 'Cavendish Enano' ranged from 25.0 to 34.9 days and those in 'Williams' were 27.0 to 36.9 days as marked between dotted vertical lines, showing a slight variation contrasting greatly with the higher values (Figure 3B, E). As shown in Figure 3C and F, lower LDNA-% values, which might be attributed to tolerance to black Sigatoka varied from 38.0 to 44.9 % in 'Cavendish Enano' and from 33.0 to 39.9 % in 'Williams'. Whereas, LDNA-% values from non-irradiated plants did not show values as low as 44.0 % in 'Cavendish Enano' and 38% in 'Williams'.

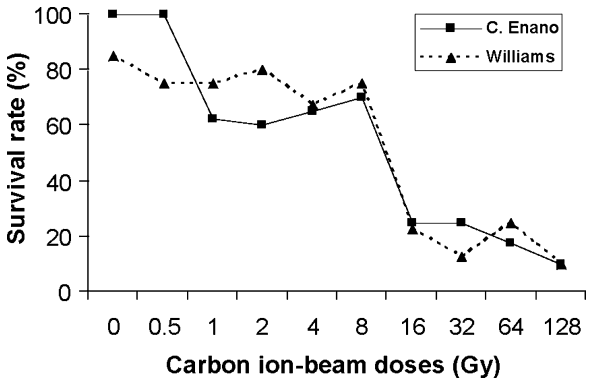

Figure 2. Survival rate (%) of the banana plantlets in 'Cavendish Enano' and 'Williams' at 19 days after ion-beam irradiation.

Using the II-% index to evaluate a hybrid population of plantain, Cohan et al. (2003) demonstrated that 'CRBP-39' (AAAB) had a character of excellent resistant to black Sigatoka in three development phases: vegetative phase at 6-month, flowering phase and harvest phase. Therefore, II-% is a very useful parameter for evaluating the resistance of plants to this disease. In this research, the II-% allowed us to observe an inter-individual variation on response to black Sigatoka, which is varying from very susceptible to tolerant among the plants regenerated from irradiated explants, when subjected to the inoculum. Probably, the effect of the irradiation could cause DNA alteration as mentioned by Fukuda et al. (2004), and result in expanding the variation in relation to this pathogen.

Lower infection reaction is closely related to the plant defense mechanism. By crossing two susceptible triploid plantain cultivars ('Bobby Tannap' and 'Obino 1 Ewai') as female parents with the resistant wild diploid banana 'Calcutta 4', Ortiz and Vuylsteke (1994) obtained segregated progenies with a durable horizontal resistance. In the case of the progenies having partially resistant response, it was observed that slow lesion development and ultimately reduced sporulation. Ortiz and Vuylsteke (1994) also discussed that the possible mechanisms of black Sigatoka resistance were expressed by different pathways such as the synthesis of phytoalexins, the production of lignin or suberin, polyphenolic content (higher in resistant cultivar), low stomata density and increased epicuticular wax. However, the mechanism is still obscure.

Leaf-disc bioassays also have been reported to be effective to evaluate resistance in several crops. Ostry et al. (1988) working with *Septoria musiva*, a disease of *Populus* spp, described that this method was sufficiently sensitive to distinguish among clones with high, moderate or low resistance. In this research, the leaf discs of banana were confronted to juglone toxin. Less affected plants were found out (Figure 1D pointed by arrow), assuming that the ion beam irradiation promoted

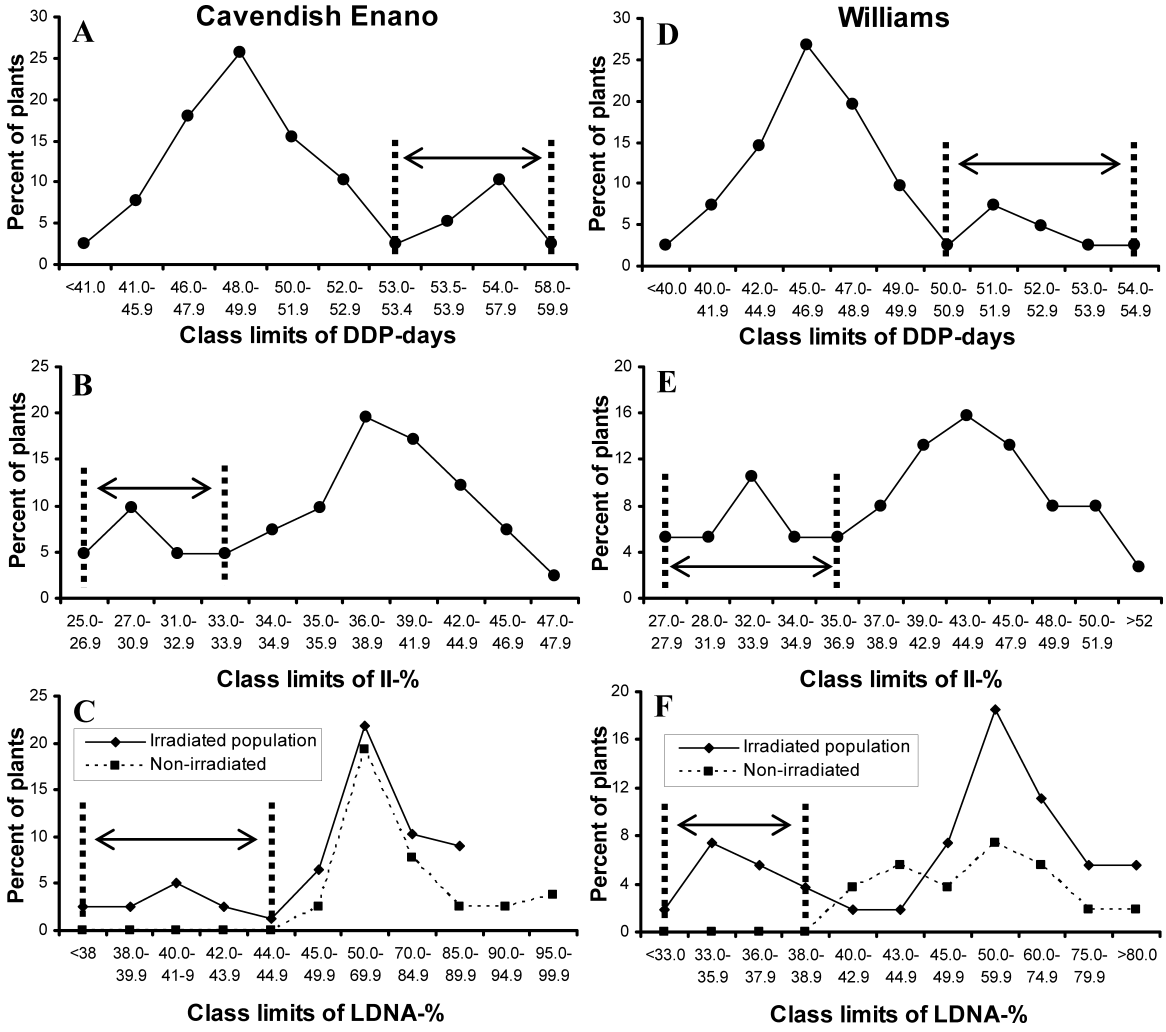

Figure 3. Efficiency of mutagenesis occurred after ion-beam irradiation analyzed by frequency distribution. Class limits range between dotted vertical lines indicates the best values of DDP-days, II-% and LDNA-% in 'C. Enano' (A–C) and 'Williams' (D–F).

mutation in cells, which results in permitting slow penetration of the toxin into the cells. Etame (2003) inoculated banana genotypes showing different reactions to black Sigatoka with juglone toxin and the pathogen for selecting resistant plants. As result, the resistant genotypes to juglone toxin ('Fougamou', 'Pisang madu', 'M53' and 'Klutuk') also showed resistant to the pathogen, although some resistant cultivars to *M. fijiensis* were susceptible to juglone. In conclusion, this method should only serve as a preliminary screening technique before field tests.

Taken the results of three parameters DDP-days, II-% and LDNA-% together, variations in relation to tolerance to black Sigatoka have been expanded in the banana population established from *in vitro* plantlets irradiated by carbon-ion beam, suggesting that ion beam is a useful tool for mutation breeding in banana.

For selecting tolerant/resistant plants, the variables DDP-days, II-% and LDNA-% were combined by a linear regression to assess the response to black Sigatoka in the irradiated materials. The linear regression permitted to categorize the plants which showed better response to this disease. LDNA-% regression versus II-% showed high significance ($P \leqq 0.01$) compared with the LDNA-% versus DDP-days and DDP-days regression versus II-% that were significant at 5% level ($P \leqq 0.05$) in 'Williams'. The regression among the three combined variables permitted us to select 6 plants in 'Williams' and 2 plants in 'Cavendish Enano' as candidates with better tolerance to black Sigatoka.

Regarding the complete assessment of the candidate plants, field experiment based on the whole plant cycle is necessary to evaluate not only the response to black Sigatoka but also fruit quality, potential production and postharvest parameters as valuable components for final selections.

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