Gamma Rays and Carbon Ion-Beams Irradiation for Mutation Induction to Breed Banana (*Musa* spp.), Especially on Response to Black Sigatoka Disease

January 2007

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A Dissertation Submitted to
the Graduate School of Life and Environmental Sciences,
the University of Tsukuba
in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy in Agricultural Science
(Doctoral Program in Biosphere Resource Science and Technology)

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Chapter 1

1. 1. Introduction

Banana (*Musa* spp.) is a worldwide extended crop growing in most of the countries located especially in the tropical and subtropical areas. FAO (2006) reported that the banana world export in 2004 was more than 15 million tones equivalent to more than $ 5 billion dollars. In the case of Ecuador, exportation in 2004 were more than 4.5 million tons from a harvested area of 216.510 ha, which represent 30% of the world export.

Bananas and plantains are valuable export products in many countries of Central and South America (Upadhyay *et al*., 1991)**,** but they are the primary food source for millions of people in many areas of the world, including Central Africa, Southwest Asia, Central and South America and the Caribbean. People in these regions are generally faced with high population growth and recurring food shortages, conditions that augment the importance of high yield, low cost crops like bananas and plantains. They yield a sweet, nutritious fruit and produce a starch that can be used to prepare a variety of food (Stover and Simmons, 1987).

Preparation methods are very diverse and vary according to the country. Bananas are eaten boiled, fried or grilled; the fruit is crushed to make an edible paste or fermented to make local beer; the burnt peel is used to make soap etc. In Côte d'Ivoire, for example, the foods made from plantains have various names of which the commonest is "foutou" which is mashed plantain accompanied by a sauce (Nkendah and Akyeampong, 2003).

In Ecuador, most of the banana production is by the commercial type Cavendish, mainly 'Cavendish Enano' (Grande Naine), 'Williams',

and ‘Valery’, which are exported to different countries. The local consumption of these cultivars are very few, consuming mostly the ‘Gros Michel’ type which are still planted in the small farms, though the cost is a little bit expensive than the commercial type, but sweetness, softening and more flavored make this cultivar to be favorite for the consumers. Another favorite cultivars is ‘Orito’, growing in humid areas and most of the plantation are organic, since the farmers cultivate with a minimum of cultural practices. This cultivar possess small finger, sweet and delicious and have high carotenoids content compared with ‘Cavendish’.

Banana is a large perennial herb with a pseudostem composed of leaf sheaths. Taxonomically, it belongs to the *Musaceae* family, genus *musa*, section *eumusa*, being a *monocotiledonaceae* (Champion, 1968), probably originating from Southeast Asia, especially from Malaysia and Indonesia (Haarer, 1965). According to the classification and origin of diversity, the genus *Musa*, is divided into four sections, the members of which include both seeded and non-seeded (parthenocarpic) types. Two of the sections contain species with a chromosome number of 2n = 20 (*Callimusa* and *Australimusa*) while the species in the other two sections (*Eumusa* and *Rhodochlamys*) have a basic chromosome number of 11 (2n = 22). The centre of diversity of the species is thought to be either Malaysia or Indonesia. Most cultivars are derived from two species, *Musa acuminata* (A genome) and *Musa balbisiana* (B genome). The majority of cultivated bananas arose from the *Eumusa* group of species. This section is the biggest in the genus and the most geographically widespread, with species being found throughout South East Asia from India to the Pacific Islands (Daniells *et al.*, 2001).

Edibility of mature fruits of diploid *Musa acuminata* (AA) came about as a result of female sterility and parthenocarpy, and such edible types would no doubt have been selected and maintained by humans.

Triploid AAA cultivars arose from these diploids, perhaps following crosses between edible diploids and wild *M. acuminata* subspecies, giving rise to a wide range of AAA genotypes. In most parts of South East Asia these triploids, which are more vigorous and have larger fruit, have replaced the original AA diploids. However; in Papua New Guinea, AA diploids remain agriculturally significant and a wide range of diversity is still found in cultivation. The diploid and triploid *M. acuminata* cultivars were taken by humans to areas where *M. balbisiana* is native (India, Myanmar, Thailand, Philippines) and natural hybridizations resulted in the formation of hybrid progeny with the genomes AB, AAB, and ABB. The Indian subcontinent is thought to have been the major centre for hybridization of *acuminata* types with the indigenous *M. balbisiana* and the region is noted for the wide variety of AAB and ABB cultivars. *M. balbisiana* is considered to be more drought and disease resistant than *M. acuminata*, and such characteristics are often found in cultivars containing a 'B' genome. Hybridization would have given rise to a wide range of edible types of banana, some of which would have survived and been multiplied under domestication (Daniells *et al*., 2001).

The section Eumusa most cultivated species/group posses different genome group such as AA (Sucrier, Pisang Jari Buaya, Pisang lilin, Inarnibal, Lakatan), AAA (Gros Michel, Cavendish, Red, Ambon, Ibota, Mutika/Lujugira, Orotava, Rio), AAAA (Pisang ustrali), AB (Ney poovan, Kamaramasenge), AAB (Iholena, Laknau, Mysore, Silk, Pome, Maia, Maoli/Popoulu, Pisang Nangka, Pisang Raja, Plantain, Nendra Padaththi, Pisang Lelat, Nadan), ABB (Bluggoe, Pisang Awak, Monthan, Kalapua, Klue Teparod, Saba, Pelipita , Ney Mannan, Peyan) and AABB (Laknau Der).

Commercial cultivars spread worldwide belong to 'Cavendish' subgroup such as 'Petite Naine', 'Grande Naine' or also well known as

‘Cavendish Enano’, ‘Williams’, ‘Valery’ and ‘Dwarf Parfitt’ (Daniells *et al*., 2001). However, Valmayor *et al*., (2000) reported that exist different banana names and synonyms of ‘Cavendish’ in Southeast Asia such as ‘Dwarf Cavendish’, ‘Enano’, ‘Giant Cavendish’, ‘Gran Enano’, ‘Robusta’, ‘Tall Cavendish’, ‘Lacatan’ and ‘Grande Naine’.

They are cultivated in over 10 million hectares of arable land worldwide. Total production was estimated in 1998 at over 88 million metric tons of which exports represented around 12 million metric tons (Marín *et al*., 2003).

It is estimated that banana world exports would reach some 13.9 million tons in the year 2010. Most of this growth would be in Latin American countries, in particular Ecuador and to a lesser extent Costa Rica and Colombia. In Asia, the Philippines projects to increase its exports, taking advantage of the growth of the Asian market. The United States of America, Japan, and the European Community are among the three most important import markets for export bananas, accounting for nearly 30 percent of global imports (FAO, 2001b).

In tropical and subtropical regions where banana crops are cultivated, intensive management has to be applied, otherwise production will be affected and the profits will diminish. However, though given the latest technologies in banana production, farmers are still faced by many problems due to the genetic constitution of the monocultured ‘Cavendish’ type which is susceptible to many pests and diseases. The most critical are the black Sigatoka caused by *Mycosphaerella fijiensis* Morelet, Panama disease (*Fusarium oxisporum*, race 1 and 4), bacteria *Pseudomonas solanacearum*, viruses such as the *Banana Bunchy Top Virus* (BBTV), *Cucumber mosaic virus* (CMV), nematodes *Radopholus similis* and weevils *Cosmopolites sordidus*. Actually, banana breeding programs are aimed at

finding resistance to these pests and diseases but not compromising quality of the fruit and traits such as parthenocarpy.

Black Sigatoka is one of the most serious diseases of banana and plantain. Classic symptoms include initial appearance of brown flecks, which enlarge to form necrotic lesions with yellow haloes and light grey centers. Lesions can coalesce and destroy large areas of leaf tissue, which results in reduced yields, and premature ripening of fruit (Jones and Maurichon, 1993). It is noted in almost all the *musacea* production regions in the world and is considered as one of the most devastating diseases affecting this crop (Aguirre *et al.,* 1999).

Black Sigatoka was first recognized in 1963 in Fiji. Subsequently, the disease was reported to be present throughout the areas around the Pacific, including Asia. In Latin America, it was identified in Honduras in 1972. In Africa, the first recorded incidence was in Gabon in 1978, in Australia this disease caused damage to the banana industry in 1924, but still the worldwide distribution is undoubtedly underestimated (Jones and Maurichon, 1993; Aguirre *et al*., 1999).

In Costa Rica, the control of this disease has totaled to more than $ 17 million/year (US). In Central America, Colombia and Mexico, it surpassed $ 350 million (US) in the last eight years. Large fruit producing companies must spray fungicide mixtures in 14 cycles annually to control black Sigatoka, spending up to 30% of their gross income (Strobel *et al*., 1993). Systemic fungicides provide effective control in commercial plantations but their effects on environment must be concerned (Jones and Maurichon, 1993). Environmental scientists have long identified the application of fungicides through spraying by airplanes as detrimental to workers and local population’s health (FAO, 2001a).

Related to Panama disease (*Fusarium oxisporum*, race 1 and 4), race 4 may prove to be an even more destructive foe than black Sigatoka.

Although it is now found only in Australia, Taiwan, South Africa and the Canary Islands, the soil-borne fungus is spreading rapidly. Race 4 is a killer disease that wipes out crops completely and cannot be controlled by existing fungicides. The only counter measure is genetic resistance (Hibler and Hardy, 1998).

Banana Bunchy Top Virus (BBTV) is one of the most serious diseases of banana. Once established, it is extremely difficult to eradicate or manage. BBTV is widespread in Southeast Asia, the Philippines, Taiwan, most of the South Pacific islands, and parts of India and Africa. BBTV does not occur in Central or South America. The virus is spread from plant to plant by aphids and from place to place by people transporting planting materials obtained from infected plants. So far, there is no cure for BBTV. Some banana varieties, like the 'Cavendish' types, are more readily infected by the virus, but no varieties of banana are resistant. Banana plants with symptoms rarely bear fruit, and because they are reservoirs of the virus, must be destroyed. The most important factors controlling banana bunchy top virus are to kill the aphid vector (disease carrier) and to rough (removing and destroying) infected banana plants (CTAHR, 1997).

BBTV infected plants with advanced symptoms have a rosetted appearance with narrow, upright and progressively shorter leaves, giving rise to the common name "bunchy top". The leaf edges often roll upwards and show a marginal yellowing. Dark green streaks are often found on the midrib and petiole, extending downwards into the pseudostem. The most diagnostic symptoms are short dark green dots and dashes along the minor leaf veins, which form hooks as they enter the edge of the midrib (Thomas *et al*., 1994).

Burrowing nematode *(Radopholus similis*) is among of the most destructive root pathogens which attacking bananas in tropical production zone. Vegetative propagation using infested corms or suckers has

disseminated this pest throughout the world. Although a number of nematode species infect bananas and plantains, *R. similis* is considered to be the main nematode problem of intensive commercial bananas, especially Cavendish types (Sarah *et al*., 1996).

Banana breeding started 70 years ago in Jamaica and Trinidad. Results from these and other breeding schemes have laid down the basics of banana breeding (Swennen and Vuylsteke, 1993). Most of the commercially grown banana cultivars are selections collected and distributed over the last two millennia. Breeding new varieties is tedious and only in recent years have breeders' selections been distributed and evaluated for farmer and consumer acceptability. Banana exporters have well-defined opinions on what is required in an ideal commercial banana variety and breeders have had difficulty in incorporating other important characteristics into varieties that are acceptable. All of the varieties of dessert bananas grown for export are similar, known as ‘Cavendish’ (*Musa acuminata* AAA). New advances in genetic engineering could enable these favored varieties to be improved through introduction of specific characters such as disease resistance without changing other attributes. There is an opinion within the international banana trade that only ‘Cavendish’ varieties have the quality that the consumer in northern hemisphere importing countries wants. Thus far consumers have not accepted bananas which taste different from ‘Cavendish’. Therefore one can expect research and development to continue its concern on ‘Cavendish’ varieties (FAO, 2001a).

For the exporter, a replacement for the pest and disease susceptible ‘Cavendish’ clones must have all the positive post-harvest attributes concerning “green-life” (how long the fruit can be held in the un-ripe state), shelf-life after ripening and consumer acceptability which really means flavor but also cosmetic attractiveness. Some of the disease resistant

varieties bred at the Jamaican programme in the 1960s and the dessert varieties of the FHIA (Fundación Hondureña de Investigación Agrícola) program do not have the enough shelf-life and flavor comparable with ‘Cavendish’ and have not been adopted as export bananas. If consumers in tasting panels have only one impression of a banana flavor, that of ‘Cavendish’, which has been the only ripe banana on sale for most peoples' life time, it is not surprising that bananas with slightly different flavors may be less favored simply because they do not taste like a “typical” banana (FAO, 2001a).

The development of the ‘FHIA-01 (Goldfinger)’ and ‘Mona Lisa’ bananas through a cross-breeding program launched in 1985 has been the most significant success in Honduras and Latin America. Working with the Honduran Foundation for Agricultural Research (FHIA) and the International Network for the Improvement of Banana and Plantain, researchers in Honduras developed and tested new hybrids of bananas and plantain that were resistant to black Sigatoka spot disease. The ‘Goldfinger’ and ‘Mona Lisa’ bananas can be cultivated in poor soil and cooler temperatures and are highly productive, growing large bunches resistant to Panama disease race 4 which attacks the most common export banana (IDRC, 2002).

According to the FAO (2003), banana is essentially a clonal crop with many sterile species, which makes progress through conventional breeding slow and difficult. Because of this, new breeding methods and tools, including biotechnology, will be helpful to develop resistant bananas for cultivation. This does not necessarily mean the use of transgenics. FAO has called for the development of more diversity in the banana, especially for export bananas; promoting awareness of the inevitable consequences of a narrow genetic base in crops and the need for a broader genetic base for commercial bananas; and strengthening plant-breeding programs in

developing countries for banana and other basic staple crops. Van Harten (1998) reported based on estimates that about one third of the total production of agricultural crops is lost due to plant diseases and pests, therefore, it would be very useful if mutation breeding could contribute to an effective pest and disease control scheme in crop plants.

Genetic improvement of banana and plantain in the various programmes operating around the world is based on crosses between commercial triploids and improved diploids, with the objective of developing higher yielding cultivars that are more resistant to the main diseases (Sigatoka disease caused by *Mycosphaerella musicola*, black leaf streak caused by *Mycosphaerella fijiensis*, fusarium wilt caused by *Fusarium oxysporum* f.sp. cubense), and pests as nematodes and weevils (Ramirez *et al*. 2005). However, the fact that triploid cultivars are seedless makes them edible but it is also a constraint when it comes to improving their yield and resistance to biotic stresses. Breeders always aim to get parthenocarpic hybrids with enhanced resistance (Krishnamoorthy, *et al*., 2004b).

The Cavendish banana is important in world trade, but accounts for only 10 percent of bananas produced and consumed globally, according to FAO (2003). Virtually all commercially important plantations grow this single genotype (Subgroup Cavendish). The Cavendish's predecessor, ‘Gros Michel’ which has been suffered the same fate at the hands of fungal diseases, prompts breeders and growers alike to find a suitable replacement for ‘Cavendish’ banana. Fortunately, small-scale farmers around the world have maintained a broad genetic pool, which can be used for future banana crop improvement.

Conventional *Musa*'s breeding are utilizing sources resistant to black Sigatoka in wild *Musa* species, especially *M. acuminata* ssp. *Burmannica,* ssp. *Malaccensis* and ssp. *siamea*, and in diploid cultivars

such as ‘Paka’ (AA) and ‘Pisang lilin’ (AA). Genetic resistance to black Sigatoka is clearly the best long-term goal for disease control especially for smallholders who cannot afford to purchase chemicals. Cultivars such as ‘Pisang Awak’ (AAB), ‘Yangambi km 5’ (AAA), ‘Pisan Ceylan’ (AAB-Mysore), ‘Pelipita’ (ABB) and ‘Saba’ (ABB) have resistance levels with good agronomic potential. However, these do not suit all local tastes and some are susceptible to *Fusarium* wilt (Jones and Maurichon, 1993). ‘Ducase’ and ‘Kluai Namwa Khom’ cultivars have been reported by Daniells and Bryde (1999) as resistant to black Sigatoka and are being utilized by the banana replacement programmes in northern Australia.

‘Calcuta 4’, belonging to the *Musa acuminata* ssp. *burmannicoides*, shows a considerable potential as a male germplasm source in breeding plantain for black Sigatoka resistance, unlike the wild types belonging to the subspecies *malaccensis*, whose defects in bunch and bunch size were transmitted to their progeny (Swennen and Vuylsteke, 1993). In Ecuador, ‘FHIA-01’ and ‘FHIA-02’ cultivars have shown a low disease index value (31.1 and 37.3%, respectively) when black Sigatoka was evaluated, in contrast with ‘Valery’ (a ‘Cavendish’ type), which showed 97.2% of disease index (Reyes-Borja, 1995).

The Global *Musa* Genomics Consortium (2002) mentioned that after rice and *Arabidopsis*, the banana is the third plant genome sequenced. Comprised of just 11 chromosomes with a total of 500 to 600 million base pairs, the banana genome is the smallest of all plants and researchers expect quick results. There are already frameworks of genetic maps based on genetic markers. Large insert DNA libraries (BACs) are becoming available, and populations of hybrids are being made for mapping and trait evaluation. Regarding resistance genes to pests and diseases, existing maps should allow isolation of genes involved in black Sigatoka resistance. Currently, a QTL for Sigatoka resistance has been anchored on a map

developed at CIRAD and could be mapped allowing isolation of genes (or cluster of genes) involved in the resistance to the disease. On the other hand bananas are the only known plant in which a virus (the Banana Streak Virus) imbeds pieces of itself into the banana's own DNA, only to pop out during times of stress, reassemble itself, and cause disease. The banana genome sequence should reveal just how this virus is able to strike when the plant is most vulnerable. It may provide a powerful new tool for targeted genetic transformation.

The continuous advance in high-throughput DNA automation and sequencing technologies has resulted in important breakthroughs in plant science. Banana with a haploid genome size of 500–600 Mbp, is among the smaller ones found within non-graminaceous monocotyledons. This characteristic turns banana into an interesting candidate for comparative genomics. Being a monocotyledon but distantly related to rice, banana could represent a useful comparison point between dicotyledonous and monocotyledonous genomes. In addition, a number of important traits, not present in model plants, can be functionally analyzed in banana. In parallel, partially and highly fertile wild diploids have also been adapted to the same environment, making banana a fascinating model by which to study both plant evolution and plant-pathogen co-evolution at a genomic level. An attractive example for the latter is the integration of the banana streak badnavirus in the plant genome, which can be reactivated after recombination. Similarly, the widespread presence of *gypsy*-like long terminal region (LTR) retroelements (200–500 copies per haploid genome and Ty1-*copia*-like retrotransposons makes a challenge for genome studies in banana (Aert, *et al.*, 2004).

Carreel *et al*., (1999) reported that the screening for resistance to black Sigatoka has led to the identification of two forms of resistance reported as High Resistance (HR, hypersensitive response) and Partial

Resistance (PR). In connection with the breeding program, a genome mapping approach for genetic and QTL analyses was developed to characterize the HR form. An F2 segregating population of 153 plants was obtained from a cross between two wild seeded banana accessions; *Musa acuminata burmannicoides* type Calcutta 4 and *M. acuminata banksii* type Madang. A linkage map was constructed with 110 AFLP markers in association with 39 codominant RFLP and SSR markers to anchor the linkage groups to the banana genetic core map. Results showed significant allelic distorted segregations in the F2 progeny for 58 markers which highlight the use of molecular studies for inheritance analyses. A first mapping concluded to join the markers in 11 linkage groups. Correlations made with field observations, ranked into 6 classes of infection severity, led to the identification of one RFLP marker strongly associated to the resistance and a second QTL mapped onto a different linkage group with lower significance level.

Because conventional breeding programs are unlikely to produce a suitable banana cultivar to replace 'Cavendish', a potential alternative, which would be the development of a genetically modified 'Cavendish' cultivar with resistance to black Sigatoka. However, public concern about genetically modified food in North America and Europe may hinder the development of this alternative (Marin *et al*., 2003). Conversely, as mentioned by FAO (2001a), consumers would have concerns regarding on the possibility that genes from such traits as resistance to herbicides or insects will escape into the local flora, damaging the environment. These are controversial issues; however, since banana plants do not produce pollen, being sterile, thus the question of dispersal into the environment of genes in transformed bananas does not arise.

Mutation breeding is characterized by its merits, which is the creation of new mutant characters and addition of very few traits without

disturbing other characters of variety (Morishita *et al*., 2001). The difficulties associated with conventional breeding led to the exploration of other techniques for introducing useful characteristics into otherwise reliable clones. Mutations can be induced with chemicals, irradiation and also by *in vitro* tissue culture. The strategy is not without difficulties of a practical nature as it involves the production and subsequent growth and observation of large numbers of individual plants from which selections are made (FAO, 2001a).

Van Harten (1998) defines mutation in higher plants as, any heritable change in the idiotypic constitution of sporophytic or gametophytic plants tissue, not caused by normal genetic recombination or segregation. Mutagenesis is also defined as the process of mutation formation at the molecular levels. Spontaneous mutation can occur without intentional human intervention as a result from the activity of so-called transposons (mobile genetic elements that can move within the genome, from one place to another and affect the activity of the gene which they are inserted). A tobacco mutant cultivar 'Chlorina', which became the first radiation-induced mutant cultivar in the world, was grown from 1936 in Indonesia since it had been released. It is often found that induced mutations may occur in frequencies of 10^3 higher than spontaneous mutations. Moreover, it is mentioned that even though mutagenesis have been occurred a long time ago in a plant, but those remained unobserved for some reason, at certain moment those may became visible, e.g. by a morphological change in a plant part, and then create the impression of a recent event. Another reason to consider using induced mutations is that breeding programmes could eventually be speeded up considerably.

Irradiation of plant materials such as seeds, buds and plantlets with gamma rays or neutrons can introduce changes in DNA sequences and rearrangement of parts of chromosomes. These changes have resulted in a

large number of improved mutant crops demonstrating, disease resistance, early maturity, drought tolerance, and better yield. Over the past 60 years, 1,800 new mutant plant varieties induced by radiation have been officially released and are now growing on millions of hectares of land. In Japan, more than 10 years ago a mutant variety of Japanese pear 'Nijisseiki' was developed by low dose-rate gamma rays irradiation method. This new variety, named as 'Gold Nijisseiki' has an excellent resistance against black spot disease. By the FAO/IAEA laboratories and the member states such as Indonesia and Malaysia, mutant varieties of banana which are more resistant to plant disease (*Fusarium*) and higher yield are being developed by radiation mutation in combination with tissue culture techniques (Machi, 2002).

In terms of radioactivity, the improved mutant crops obtained by gamma rays are not dangerous for consumption by human beings, and on the contrary, is useful even for production of safe food. Most food irradiation facilities utilize the radioactive element ^{60}Cobalt as a source of high energy gamma rays. These gamma rays have sufficient energy to dislodge electrons from some food molecules, thereby converting them into ions (electrically charged particles). Gamma rays do not have enough energy to affect the neutrons in the nuclei of these molecules; therefore, they are not capable of inducing radioactivity in the food. ^{60}Cobalt is usually the preferred source of radiation for food. Irradiation dosage is a function any energy of the radiation source dependent upon the time of exposure. Doses are usually expressed by kiloGrays (kGy); 1 Gray is equivalent to 1 joule of absorbed radiation/kg tissue (Doyle, 1999).

As mentioned above, gamma rays at present are the most favored mutagenic agent, having no particles and electric charge. However, their great penetrating power makes them dangerous as they can cause considerable damage when they pass through the tissue. The distance

between the source and the irradiated plants determines the dose rate applicable. Chronic irradiations in general lead to a somewhat lower mutation frequency than the same total dose when it is administered as an acute irradiation. Gamma sources can be used to irradiate a wide range of plant material like seeds, whole plants, plant parts, freshly picked flowers on agar, anthers, pollen grains, single cell cultures or protoplasts. Cell cultures of higher organisms which consist of haploids cells show a ten times higher radiation sensitivity than diploid cells (Van Harten, 1998).

Novak *et al*. (1990) in their research about mutation induction of banana and plantain found out that after irradiation of excised shoot-tips, *Musa* cultivars exhibited significant differences in radio sensitivity and post-irradiation recovery assessed as fresh weight increase. These differences were dependent on the ploidy level and the hybrid constitution by genomes A (*acuminata*) and B (*balbisiana*). A suitable exposure of *Musa* shoot-tips cultured *in vitro* as doses 20-25 Gy seem recommendable for diploid SH-3142 and doses 30-35 Gy are suitable for 'Grand Nain', 'Pelipita' and 'Saba'. The highest dose 35-40 Gy of gamma irradiation was found suitable for mutation induction in 'Highgate' and tetraploid 'SH-3436'. The frequency of phenotypic variation ranged 30-40% of tested M_1V_4 plants, dependent on genotype and irradiation dose. One early-flowering plant ('GN-60 Gy/A') was identified among the population of 'Grand Nain' regenerated from shoot-tips irradiated with 60 Gy. This plant grew vigorously and began flowering after nine months in comparison with 15 months in the non-irradiated control. The same flowering time has been observed for the second and third pseudostems. In terms of protein analysis, the protein banding pattern was different between the original 'Grand Nain' and the mutant. Probably the most prominent difference was in the intensity (quantity) and mobility of a major protein having a molecular weight of about 33 kDa. The original clone showed a densely stained band

which migrated faster (Rf = 0.44) than that of the mutant ‘GN-60 Gy/A’. In addition, three other bands were not observed in the mutant, but only in the original ‘Grand Nain’. Such bands were less densely stained with and Rf value of 0.19, 0.31 and 0.64 and molecular weight of about 94 and 26 kDa, respectively (Novak *et al*., 1990).

Regarding to the tissue culture, this technique has revolutionized banana cultivation and has replaced the use of conventional vegetative suckers in many of the intensive banana-growing regions. It is estimated that up to 50 million tissues cultured plants are produced annually. The use of tissue culture planting material can prolong the pest-free period for months or possibly years. The ubiquitous pests *Radopholus similis,* the burrowing nematode and *Cosmopolites sordidus,* the banana weevil are examples of problems that have arrived with the conventional vegetative suckers. The problem of viruses has not been solved by tissue culture and the international movement of some germplasm it will continue restricted until development of satisfactory methods of virus elimination. Banana streak virus (BSV) is a particular problem as it cannot be eliminated by the conventional techniques of heat treatment or apical tip culture (FAO, 2001a). However, Novak *et al.* (1990) have mentioned that *in vitro* culture of split shoot tips in liquid medium supported a high degree of shoot proliferation. Every 30 days, approximately 6-8 new shoots could be separated from a multiple shoot clump. This technique allowed the preparation of a sufficient amount of shoot-tips as test units for mutagenic treatment.

The use of the floral axis tip from a “mother plant” versus vegetative apices from lateral buds of the same plant as a source of the primary explants were compared and contrasted. Materials from floral axis tip consistently showed high phenotypic uniformity whereas materials from vegetative apices of “sword” suckers were less so. “Virus-like symptoms”

that became apparent in much of the material just before flowering (shooting stage) were determined to be due to the Banana Streak Virus (BSV), a dsDNA pararetrovirus. The “good news” is that a primary explants taken from the floral axis tip was quicker in its initial response to yield a multiplication system *in vitro*, and produced significantly fewer virus-infected plants, ca. 5%. By contrast, primary explants obtained from the vegetative sucker-derived apices were later in their production of initial buds, and produced many more virus-infected plants with an average of 32% (Krikorian *et al*., 1999).

In the other hand, tissue culture as a biotechnological technique became useful for banana screening in the earlier stages of plant growth, since banana is a perennial herb. In the past, evaluations at field conditions were tedious and time-consuming process. For example just for black Sigatoka, using traditional methodology, mature plants are challenged by fungal spores produced on naturally infected leaves according to fastidious inoculation schemes to artificially induce the disease. This process may require over 12 months establishing unequivocally the susceptibility or resistance of a particular cultivar to the disease (Stover, 1986). Actually, there are availability of techniques applicable in tissue cultured plants for different kind of selections, making easy screenings and reliable for breeding bananas. Selections techniques such as aluminium-tolerant variants (Matsumoto and Yamaguchi, 1988; Matsumoto and Yamaguchi, 1990), *in vitro* selection of *M. fijiensis*-resistant using a host specific toxin or crude extracts (Okole and Shulz, 1997; Strobel *et al*., 1993; Hoss, 1998; Hoss *et al*., 2000; Molina and Krausz, 1988) and *in vitro* screening for *Radopholus similis* and *Fusarium oxisporum* (Elsen *et al*., 2002; Severn-Ellis *et al*., 2003) have been successively used to breeding banana.

One of the most famous allelopathic plants is black walnut (*Juglans nigra*). The chemical responsible for the toxicity in black walnut

is juglone (5 hydroxy-1,4-napthoquinone) and is a respiration inhibitor. *Solanaceous* plants, such as tomato, pepper, and eggplant, are especially susceptible to juglone. These plants, when exposed to the allelotoxin, exhibit symptoms such as wilting, chlorosis (foliar yellowing), and eventually death. Other plants may also exhibit varying degrees of susceptibility and some have no noticeable effects at all. Some plants that have been observed to be tolerant to juglone include lima bean, beets, carrot, corn, cherry, black raspberry, catalpa, virginia creeper, violets, and many others. Juglone is present in all parts of the black walnut, but especially concentrated in the buds, nut hulls, and roots. It is not very soluble in water and thus, does not move very rapidly in the soil. Toxicity has been observed in all soils with black walnut roots growing in it (roots can grow 3 times the spread of the canopy), but is especially concentrated closest to the tree, under the drip line. This is mainly due to greater root density and the accumulation of decaying leaves and hulls (Rivenshield, 2003).

It is already well-known that the *M. fijiensis* has seven compounds of phytotoxins in which the biological activity of each of the compounds isolated displayed phytotoxicity at various test concentrations on one or more cultivars of banana or plantain. An experiment carried out by Stierle *et al*. (1991) determined that 2, 4, 8-trihydroxytetralone ($C_{10}H_{10}O_4$), exhibited the greatest host selectivity, analogous to the fungal pathogen itself, particularly at the 5µg/5µL level. A black Sigatoka-resistant cultivar (‘IV-9’) was insensitive to 2, 4, 8-trihydroxytetralone up to 10µg/5µL application rate in the leaf bioassay test. ‘Saba’, a tolerant banana cultivar, was only slightly reactive to 2, 4, 8-trihydroxytetralone, unlike the extremely disease-susceptible varieties ‘Bocadillo’ and ‘Horn plantain’ which developed large necrotic lesions after application of toxins. This compound shows definite potential as a screening tool for toxin

sensitivity in tissue culture systems. Juglone ($C_{10}H_6O_3$) was the most biologically active phytotoxin, which induced the formation of necrotic lesions on all cultivars of banana and plantains at 0.1 µg. 2-carboxy-3-hydroxycinnamic acid ($C_{10}H_8O_5$), a novel compound demonstrated some host selectivity at the 5 µg 5µL^{-1} level which again paralleled that of the fungus. Isoochracinic acid ($C_{10}H_8O_5$) and 4-hydroxyscytalone ($C_{10}H_{10}O_4$) were considerably less active than the other phytotoxins isolated and displayed no host selectivity. Fijiensin, previously reported as a phytotoxins from the fungus, exhibits a low level of bioactivity, and host selectivity.

Toxins of *M. fijiensis*, have potential usefulness in plant breeding/selection programs in all parts of the world where banana and plantains are grown. The fungal pathogen causing black Sigatoka disease, attacks almost all varieties of cultivated bananas and plantains *Musa* spp. However, neither the reactions of host plant in relation to the pathogen nor its pathogenicity has been characterized in detail. A special significance has been attributed to fungal secondary metabolites as host-specific toxins within the pathosystem. Using *in vitro* conditions, the metabolites flaviolin, 2-hydroxyjuglone, juglone and 2, 4, 8-trihydroxytetralone (2, 4, 8-tht) had been determined. The results proved the importance of 2, 4, 8-tht for the development of necrotic leaf symptoms that causes host-specific reactions depending on their concentration at different moments of pathogenesis (Hoss, 1998).

Cadet *et al*., (1995) with a short survey of the main available information on the molecular mechanisms of action of heavy ions on DNA have assumed that nucleic acids constitute one of the major targets of the deleterious effects (cell inactivation, mutation) of cosmic radiation and particularly charged heavy particles (Z>2) having a linear energy transfer (LET) higher than 50 keV/pm. This may be inferred mostly from ground

experiments involving various biological materials. In particular, the induction of chromosomal aberrations, mutations and neoplastic transformations was observed in various metabolically active cells from yeast and mammals. When high-frequency electromagnetic radiation or very penetrating heavy charged particles interact with biological material, a great deal of energy is dissipated in the cell. In addition, the interaction of ionizing radiation free or bound water molecules leads to the generation of reactive oxygen species such as hydroxyl radicals. The radical reactions arising from the direct and indirect effects of gamma radiation or X-rays lead to various chemical modifications, which, in the case of DNA model compounds, are at least partly known: single- and double-strand DNA breaks, purine and pyrimidine base lesions, DNA-protein cross-links. However, it should be noted that the bulk of the modifications arising from exposure of cellular DNA to sparsely ionizing radiation remains, for the most part, unknown. The use of high LET radiation provided by accelerators has allowed investigations of DNA damage at the cellular level. In particular, DNA double-strand cleavages and chromatin breaks have been measured by using sensitive techniques in bacterial and mammalian cells. Induction of DNA double-strand breaks in cells has been found to be linear with dose for all heavy ions investigated.

Ottolenghi *et al*. (1999) in their studies about chromosome aberration induction by ionizing radiation mentioned that a large number of experimental data sets showed visual chromosome aberrations at the first post-irradiation mitosis due to chromosome breakage and illegitimate reunion of the pieces (misrejoining or misrepair) if cells are exposed to ionizing radiation during the G_0/G_1 phase of the cycle. If all chromosomes are stained with the same color (Giemsa staining), the following three main categories of aberrations are detectable: (i) dicentrics, produced by two breaks on two distinct chromosomes (inter- chromosome exchanges); (ii)

centric rings, originated from two breaks on the same chromosome, each of them on a distinct chromosome arm (inter-arm intrachanges); (iii) deletions, which can be produced either by a single unrejoined break (terminal deletions, which appear as linear acentric fragments), or two breaks on the same chromosome arm (interstitial deletions, which are scored as small paired dots). The more recent fluorescence *in situ* hybridization (FISH) technique, which allows selective painting of specific pairs of homologous chromosomes, has made it possible to identify a larger number of aberration types, including translocations (which are symmetrical interchanges and cannot be detected with traditional Giemsa staining) and complex exchanges usually defined as chromosome exchanges involving three or more breaks and two or more chromosomes.

Fukuda *et al*. (2003) reviewed the molecular mechanism of mutation by ion beams in order to investigate the DNA alteration of mutation induced by ion-beams in plants, polymerase chain reaction (PCR) and sequencing analysis to compare DNA fragments were performed for amplified carbon ion- and electron-induced *Arabidopsis* mutant. Fourteen out of 30 loci possessed intragenic mutation ("small mutation") such as point mutation or deletion of several to hundreds of bases. For comparison, 16 out of 30 loci possessed intergenic mutation ("large mutation"), such as chromosomal inversion, translocation, and total deletion covering their own loci. These results imply that in the case of mutation induced by ion beams, half of the mutants have intragenic small mutation (47%) and the other half have large DNA alteration such as inversion, translocation (40%), and large deletion (13%). In such an alteration, a common feature was that all the DNA strand breaks induced by carbon ions were found to be rejoined using short homologies.These results suggest that the nonhomologous end joining pathway operates after plant cells are exposed to ion beams.

Tanaka (1999) reported novel genes in *Arabidopsis*, by using ion beam irradiation. The *ast* and *sepl* mutants were obtained from the offspring of 1,488 carbon ion-irradiated M_l seeds respectively. The *uvi1-uvi4* mutants were also induced from 1,280 M_1 lines. Thus, ion beams can induce not only known mutants such as *tt*, *gl* and *hy* also novel mutants with high frequency. Even in the *tt* phenotype, was found two new mutant loci other than know loci. Also in chrysanthemum have been produced several kinds of single, complex or striped flower-color mutants that has never induced by gamma irradiation, indicating that ion beams could produce variety of mutants on the same phenotypes. In conclusion, the characteristics of ion beams for the mutation induction are 1) to induce mutants with high frequency, 2) to show broad mutation spectrum, and 3) to produce novel mutants. For these reasons, chemical mutagen such as EMS and low LET ionizing radiation such as X-ray s and gamma rays will predominantly induce many but small modifications or DNA damages on the DNA strands, resulting in producing several point like mutations on the genome. On the contrary, ion beams as a high LET ionizing radiation will cause not so many but large and irreparable DNA damage locally, resulting in producing limited number of null mutation. Ion beam-induced mutation will be useful to produce novel and null mutation with high frequency for the coming functional genomics. Furthermore, as ion beams could produce mutant with large DNA alteration such as deletion and inversion.

Shikazono *et al.* (2003) reported novel *tt* mutants in *Arabidopsis thaliana* induced by ion-beams. They demonstrated from the present study that the frequencies of embryonic lethal and chlorophyll-deficient mutants induced by carbon ions with 11-fold and 7.8-fold higher, respectively, than those induced by electrons and that the mutation rate per Gray of carbon ions (1.9×10^{-6}/Gy) was 17-fold higher than that of electrons. It is known that carbon ions have a LET 500-fold higher than that of electrons. The

high frequency of embryonic lethal and chlorophyll mutants and the high mutation rate after carbon-ion irradiation indicate that damage produced by a single carbon ion is more mutagenic than that produced by 300 tracks of electrons. Since all loci, except *tt7* and *gl3*, were found to be mutated by carbon-ion irradiation, it is likely that carbon ions could randomly mutate the genome.

1. 2. General objectives:

In the current study the general objective are as below.

1. A study on mutation induction by gamma rays to breed banana (*Musa* spp.) coupled with *in vitro* techniques, in order to identify genetic variability, sensitivity to gamma irradiation and black Sigatoka tolerant/resistant banana mutant by using juglone toxin inoculation at young stages.

2. A study on mutation induction on banana (*Musa* spp.) by using carbon ion-beams irradiation technique and identification of black Sigatoka resistant/tolerant mutants, aiming at finding the critical ion-beams doses, genetic variability for mutant selection and; the response of black Sigatoka disease on the irradiated plants.

Chapter 2

2. 1. Materials and Methods

This section comprises the similar materials and methods that were used in both “Gamma rays” and “Carbon ion-beams” irradiation methods. For a better understanding of the general methodology, a schematic drawing is shown in Fig. 1.

2. 1. 1. Cultivars of banana used for gamma rays and carbon ion beams irradiation

Four cultivars of banana (Fig. 2) were used in both gamma rays and carbon ion-beams irradiation methods as follow.

2. 1 .1. 1. Orito

‘Orito’ *(Musa acuminata* AA), is a diploid type susceptible to black Sigatoka with an average height of 4-5 m, characterized by small fingers and bunch, and good fruit quality. In Indonesia, this cultivar is known as ‘Pisang mas’ and is popularly known as ‘Orito’ in Ecuador, as well as ‘Baby banana’ in the organic banana market. Organic ‘Baby banana’ is obtained as result of a production system sustainable in time, and optimum management of natural resources and organic sub products, minimizing external inputs use and avoiding chemicals and fertilizer use. In Ecuador, there are approximately 403 hectares cultivated with organic ‘Baby banana’. Organic Baby banana differs from banana by its large, wide and brilliant leaves. Producers have learned to deal with black Sigatoka disease without decreasing production employing defoliation of infected

leaves and throwing away the damaged parts. Organic ‘Baby banana’ is a product with high growing expectations, and at present its production is steadily increasing. Main destinations of the ecuadorian ‘Baby banana’ are the U.S.A (New York, Boston, Miami, New Jersey and Los Angeles), Europe (Holland, England, France and Germany) and Japan (PROJECT CORPEI-CBI, 2003).

2. 1. 1. 2. Williams

‘Williams’ (*Musa acuminata* AAA), is a triploid type belonging to the Cavendish subgroup, possess a normal plant with a good bunch, good fruit quality, but high susceptibility to black Sigatoka. In 1980 ‘Williams’ occupied 14% of all commercial plantings, 40% in 1985 and to 56% in 1990. Thus, ‘Williams’ is currently the predominant commercial cultivar comprising around 56% of the total plantings (ARC-Institute for Tropical and Subtropical Crops).

2. 1. 1. 3. Cavendish Enano

‘Cavendish Enano’ (*Musa acuminata* AAA), belongs to the Cavendish subgroup, having a small plant with a normal bunch and good quality fruit. This triploid type cultivar is susceptible to black Sigatoka. Currently this cultivar is becoming the most important Cavendish sub-group cultivar world wide. ‘Cavendish Enano’ known also as ‘Grand Nain’ are planted in the subtropics of South Africa, Israel and Canary Islands; but are also well known in Central and South America, Asia and Pacific (ARC-Institute for Tropical and Subtropical Crops).

2. 1. 1. 4. FHIA-01

‘FHIA-01’(*Musa acuminata* AAAB) is a tetrapliod hybrid, and very resistant to black Sigatoka. Bunch weight ranges between 39-56 kg, with a number of functional leaves at harvest stage ranging from 9 to 11. Plant height is around 4 m, with finger number ranging from 170 to 229. The fruits, however, possess a much different taste than the ‘Cavendish’ type (Reyes-Borja, 1995). ‘FHIA-01’ resists the diseases that are devastating plantations throughout the tropics. It also grows well in poor soils and at cooler temperatures, offering a promising banana in terms of extending into semi-tropical or upland areas where heat- and humidity-loving varieties cannot grow at present. After decades of painstaking breeding work, ‘FHIA-01’ or otherwise known as ‘Goldfinger’, is the first banana variety ever bred that could replace the standard ‘Cavendish’ banana. It may well save the world's banana export industry from collapse as diseases take an insurmountable toll. More important yet, it could ensure reliable food supplies for the millions of people in Africa, Asia and Latin America for whom bananas and plantains are staple foods. During breeding this cultivar, the first big breakthrough came in 1977 with the development of a hybrid that was resistant to burrowing nematodes, a widespread pest controlled by potent, expensive pesticides and race 4 of Panama disease, with a good bunch size. Crossed with a female Brazilian apple-flavoured ‘Dwarf Prata’ clone, it showed good resistance to black Sigatoka, thus, ‘FHIA-01’ was born (Hibler and Hardy, 1998).

FHIA-01 has been included in this experiment to compare pathogen-related reaction for black Sigatoka among the susceptible cultivar since that FHIA-01 is resistant to this disease and to study its sensitivity to gamma rays and ion-beams.

2. 1. 2. Plant materials and explants conditioning for gamma rays and carbon ion-beams during pre- and post-irradiation

The “Gamma rays” irradiation was carried out in collaboration with the National Institute of Radiation Breeding, National Institute of Agrobiological Science, located in Hitachiohmiya, Ibaraki Prefecture, Japan, using the facilities of “Gamma Room”, “Gamma Greenhouse and “Gamma Field”. Related to the research on “Carbon-ion-beams”, the irradiation was conducted on June 6, 2005, at the Takasaki Ion Accelerators for Advanced Radiation Application (TIARA), Japan Atomic Energy Agency (JAEA), Japan. The carbon ions with the total energy of 320 million electron volts (MeV) were generated by an Azimuthally Varying Field (AVF) cyclotron. The physical properties of the carbon ions are as follows: the incident energy at the target surface is 311 MeV (25.9 MeV/u), the range of the ions in a target is 2.2 mm, and the mean linear energy transfer (LET) in a target is estimated to be 137.6 keV/μm. After irradiation, the banana materials were brought to the Laboratory of Pomology of University, Tsukuba in order to study the effects of the irradiation.

Banana germplasm were propagated in aseptic conditions in a laminar flow chamber. Starting material has been from *in vitro* plants that were propagated before establishment of the irradiating experiment by “Gamma rays” (“Gamma Room”, “Gamma Field” and “Gamma Greenhouse”) and “Carbon ion-beams irradiation”.

2. 1. 2. 1. “Gamma rays (Gamma room)” and Carbon ion-beams irradiation

Four-week old shoot tips from the four banana cultivars were used as a source of explants for *in vitro* propagation in both “Gamma rays

(Gamma room)" and "Carbon ion-beams" irradiation methods. These shoot tips were developed in a solid medium that consisted of 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.

For fast propagation, during pre- and post-irradiation, the explants were transferred to the multiplication liquid medium by dividing the corm of each in the case of "Gamma rays (Gamma room)". The multiplication medium was same as the initiation medium but without agar.

For "Carbon ion-beams" irradiation, banana explants as thin as 2 mm were requested to allow total penetration of the ion beam. Prior to irradiation, from April 28 to May 10, 2005, an experiment was conducted to clarify the regeneration rate of the thinner banana explants. Cultivars 'FHIA-01' and 'Williams' were used. Two types of slicing or cutting methods were applied to the banana corms shoot tips (both vertical and horizontal slices). The slices were placed in liquid medium for regeneration, containing MS medium supplemented with BA (5 mg L^{-1}), and sucrose (20 g L^{-1}), at pH 5.6. Twenty-five slices were placed into a 300 mL Erlenmeyer flask containing 100 mL of medium. Five flasks (replications) containing the explants were prepared. The explants were stirred in a shaker at 100 rpm. Regeneration rate (%) and weight of explants were recorded. From the results, the highest number of regenerated plants obtained using either of the two methods were selected and applied in the establishment of the experiment for ion-beams irradiation.

In both "Gamma rays (Gamma room)" and "Carbon ion-beams" irradiation methods, the multiplication medium containing the explants was stirred in a shaker at 100 rpm and in all maintenance stages, temperature was kept at 27^{o}C. After irradiation, initiation solid medium, multiplication liquid medium and regeneration solid medium were used to propagate the explants into a largest population to study the mutations.

2. 1. 2. 2. “Gamma rays (Gamma field)”

Chronic irradiation was done using “Gamma Field” facilities. The experiment was carried out using pots 40 cm diameter containing acclimated plantlets, around 20 cm height. After irradiation, for *in vitro* culture, the meristem of each plant was cut into several explants and placed into a multiplication solid medium. Explants from this experiment were propagated three times.

2. 1. 2. 3. “Gamma rays (Gamma greenhouse)”

Chronic irradiation of banana plants was done in “Gamma Greenhouse” facility. Banana plants were irradiated for a 9-month duration, from September 2002 until the end of June 2003. To propagate the plants after irradiation, initiation solid medium, multiplication liquid medium and regeneration solid medium were used to increase plant numbers for post-irradiation studies.

2. 1. 3. Relative DNA content measured by using flow cytometer

To analyze the relative DNA content of banana leaves samples, PAS Flow Cytometer (Partec) was utilized which is equipped with a mercury arc lamp that is suited for analysis of samples stained with CyStain UV kit. The Relative Nuclear DNA Content (RDC) was calculated using the peak mean value obtained from analyzed sample (FI) divided for the peak mean of the internal standard (FI). Resulting values of RDC were analyzed by frequency distribution. Analysis for gamma-rays irradiated plants was carried out using 20 plants per each doses in ever cultivars including the non irradiated, using ‘Orito’ as standard. Only the plants

resulting from “Gamma Room” experiment were analyzed by frequency distributions. Regarding to the ion-beams irradiated samples, 115 samples were analyzed using ‘FHIA-01’ as the standard.

2. 1. 4. Factor of effectiveness

A factor of effectiveness (FE) was calculated to compare the efficiency of the mutagens using the data obtained from phenologic and phenotypic variations. The formula to calculate the FE is a modified formula by Walther (1969) cited by Bhagwat and Duncan (1998) described as follow:

$$FE = \frac{\text{Total number of variations}}{\text{Total number of plants treated}} \times 100$$

2. 1. 5. Statistical analysis

The statistical one-way analysis of variance by Tukey-Kramer (JMP, Version 5) was used to analyze data from gamma rays. For carbon ion-beams, the data were processed using the analysis of variance (General AOV/AOCV, analytical software Statistix for windows version 2.0), followed by Tukey analysis ($P \leqq 0.05$). The radiosensitivity was evaluated as survival rate-lethal doses (LD_{50}) for each. LD_{50} calculation using survival rates (%) were analyzed by exponential regression determined in the four cultivars. LD_{50} is a value to assess acute toxicity, which determines the amount of Gy necessary to kill half of the irradiated population.

Chapter 3

Mutation induction by gamma rays to breed banana (*Musa* spp.) coupled with *in vitro* techniques

3. 1. Introduction

From the middle of the last century up to now, tissue culture techniques have been very useful, significantly speeding up the breeding programs worldwide as well as opening opportunities in obtaining genetically improved germplasm with excellent characteristics. Biotechnology gave rise to many methodologies applicable at the earlier stages of plant growth, which makes suitable for mutant selection at the tissue culture level and green house conditions (Novak *et al*., 1990; Sackston and Vimard, 1988; Morishita *et al*., 2001; National Institute of Agrobiological Sciences, 2002; Van Harten, 1998; Bermúdez *et al*., 2000), making evaluation easier and more efficient. Screening for such traits as aluminum tolerance (Matsumoto and Yamaguchi, 1990), resistances to *Fusarium oxisporum* f. sp. cubense and *Radopholus similes* (Bhagwat and Duncan, 1998; Severn-Ellis *et al*., 2003; Speijer and De Waele, 1997; Elsen *et al*., 2002), *M. fijiensis* using artificial inoculation techniques (Jácome and Schuh, 1993) or its phytotoxins (Hoss, 1998; Hoss *et al*., 2000; Okole and Shulz, 1997; Stierle, 1991; Harelimana *et al*., 1997; Molina and Krausz, 1988). At the same time, genetic variation was noted when tissue culture techniques were used and to a greater extent when these variations were induced by the use of mutagens (García *et al*., 2002; Bermúdez *et al*., 2002).

In banana, many biotechnological techniques have been used for breeding especially the commercial Cavendish types that are widely accepted worldwide. However, this type of banana is characterized by such

traits as female sterility and parthenocarphy which make conventional breeding difficult (FAO, 2003). Thus, the use of radiation breeding using gamma rays could prove to be a viable method in banana breeding work. The use of mutagens has been acknowledged as a reliable method of breeding plants with improved characteristics in many crops. In this study, nuclear techniques especially the use of ^{60}Co and ^{137}Cs as sources of gamma rays was coupled with *in vitro* techniques, to induce mutation as a source of genetic variability for possible improved and more desirable traits.

This research has the following objectives: 1) to apply both tissue culture and gamma rays irradiation techniques to induce genetic variability for mutant selection, to determine the sensitivity to gamma irradiation, and select suitable dosage application to irradiate banana, 2) to conduct studies on irradiated materials aiming at finding a black Sigatoka-tolerant or resistant banana mutant, using toxin inoculation techniques in determining disease-resistance at young stages of banana *in vitro* and, 3) to identify aneuploid variants among the irradiated materials.

3. 2. Materials and Methods

3. 2. 1. Irradiation of the banana germplasm using ^{60}Co and ^{137}Cs as source of gamma rays

The experiments were established using three kinds of facilities, where different dosage and treatment were applied as mentioned below.

3. 2. 1. 1. Acute irradiation (^{60}Co) at the "Gamma Room" in banana explants

The first experiment using an acute irradiation was conducted in a "Gamma Room" (Fig. 3, A). Banana explants were irradiated using 20 explants/dish (90mm diameter) in different doses of gamma rays. The doses were: 0 (control), 50, 100, 150, 200, 300, and 500 Gy (two replications). Doses were applied 6 days after embedding the explants in the solid medium. Finally, percent survival of plantlets was determined by counting the surviving of explants, and evaluating the length and weight of the shoots one month after irradiation.

3. 2. 1. 2. Chronic irradiation (^{60}Co) at the "Gamma Field" in banana plants

Chronic irradiation was done at "Gamma Field" facilities (Fig. 3, B). The experiment was carried out using pots 40 cm diameter containing acclimated plantlets, around 20 cm height. The doses were 0.5, 1 and 2 Gy. Plants were irradiated for 34 days (from September 25 until October 29, 2002). One plant per treatment and per cultivar was used, except the cultivar 'Orito' where the dose 1 Gy was omitted. After irradiation, for *in vitro* culture, the meristem was cut into several explants and placed into a multiplication solid medium. Explants from this experiment were propagated three times.

3. 2. 1. 3. Chronic irradiation (^{137}Cs) at the "Gamma Greenhouse" in banana plants

Chronic irradiation of banana plants was done in "Gamma Greenhouse" facility. Banana plants were irradiated for a 9-month duration, from September 2002 until the end of June 2003 (Fig. 3, C). In this case the source of gamma rays was ^{137}Cs. Plantlets were irradiated in three doses: 0.25, 0.50 and 0.75 Gy (20 hours/day). Unique characteristics observed in the mutants were evaluated.

3. 2. 2. Juglone toxin screening

The materials from three multiplications after irradiation at "Gamma Room", and "Gamma Field" ("Gamma Greenhouse" irradiated plants were not screened) were acclimated under greenhouse conditions at the University of Tsukuba. The plantlets were transplanted into the small plastic pots with organic soils. When the plants reached 15-20 cm height and 4-6 leaves, inoculations were made with juglone as host-specific toxin.

Leaf-discs were obtained from the second expanded leaf with a 6 mm of radius (113.09 mm^2). 'FHIA-01' (black Sigatoka resistant) was included as resistance indicator (10.79±2.13 mm^2) as criteria to compare the irradiated susceptible cultivars ('Cavendish Enano', 'Williams' and 'Orito'). Ten mL of juglone solution-150 ppm (Reyes-Borja *et al*., 2005) were placed in a 30 mm Petri dish, embedding 12 leaf discs and kept for 24 hours in an incubator at 26^{o}C under light conditions. Leaf discs photos were obtained using a Camedia Digital Camera C-5050 Zoom (Olympus). Necrotic area was measured with the GIMP 1.2 software by counting the number of pixels per mm^2 of the full disc (113.09 mm^2) and number of pixels of the green area by selecting hand-draw regions, obtaining the value of necrotic area by difference. A total of 208, 179 and 307 plants were used

for screening in ‘Cavendish Enano’, ‘Williams’ and ‘Orito’, respectively. Data were analyzed by frequency distributions.

3. 2. 3. Putative mutants of FHIA-01

After analysis of the relative DNA content, among the plants irradiated with the higher doses (200-300 Gy), plants containing diminished DNA were selected. The plants were kept in big pots with soils and later on they were transferred to the greenhouse and kept under control temperature. Among the selected plants, two plants have different patterns of growth that will be described in the results.

3. 3. Results and Discussion

3. 3. 1. Acute irradiation (^{60}Co) at the “Gamma Room” in banana explants

Irradiation effect on plantlet weight had a similar effect on the height across all cultivars when the radiation doses increased. Doses higher than 150 Gy, strongly affected the weight in these plants (Fig. 4, A). According to the Tukey-Kramer analysis (Table 1), the four cultivars showed significant differences ($P \leqq 0.01$). Figs. 5, 6, 7 and 8 show how irradiation dose rate significantly depressed the growth of the explants when irradiation dosage was increased.

The height of plantlets decreased in all cultivars when the dose rates increased (Table 2). The effect was more pronounced in ‘Cavendish Enano’ even at the lowest dosage, resulting in high mortality when more than 150 Gy was applied. ‘FHIA-01’ at 200 Gy dose still showed vigorous growth, but this was reduced strongly when dosage was increased up to 500

Gy. ‘Williams’ and ‘Orito’ could not tolerate radiation dosages more than 200 Gy. However, the height in ‘Williams’ remained similar even when doses were increased to 50, 100 and 150 (Fig 4, B). Difference between ‘Cavendish Enano’ and ‘FHIA-01’ was significant at the 5% level ($P \leqq 0.05$), and those between ‘Williams’ and ‘Orito’ was significant at the 1% level ($P \leqq 0.01$). ‘Cavendish Enano’ across dosages was significantly difference ($P \leqq 0.05$).

Fig. 4, C shows the relationship between survival rate and doses for each cultivar. The survival ability decreased across the dosage. The survival rate of ‘Cavendish Enano’, ‘FHIA-01’ and ‘Orito’, at 50 Gy was significantly different from that of ‘Williams’ with higher survival rate (Table 3). However, when dose was increased to 100 Gy, ‘Cavendish Enano’ had significantly higher survival rate than ‘Williams’, ‘FHIA-01’ and ‘Orito’. At 150 Gy, the survival rate in ‘Williams’ remained high, while it was still low in ‘Cavendish Enano’, ‘FHIA-01’ and ‘Orito’. Above 200 Gy, ‘FHIA-01’ showed a relatively high survival rate, over the rest of the cultivars tested. A dosage of 500 Gy resulted in the death irrespective of cultivars used, except in ‘FHIA-01’.

Fig. 9, A and B, (‘Cavendish Enano’ and ‘Williams’, respectively) and Fig. 10, A and B (‘Orito’ and ‘FHIA-01’, respectively) show the survival rate (%) for LD_{50} as an analysis to assess acute sensitivity in the four cultivars. LD_{50} was highest in ‘Williams’ (83.94%), and lowest in ‘Orito’ (65.0%). Plus/minus (±) 5% was aggregated to each LD_{50} values of each cultivar to select the optimum irradiation doses of Gamma rays. Subsequently, the optimum doses for ‘Cavendish Enano’ ranged from 74.0 to 81.8 Gy, ‘Williams’ from 79.7 to 88.1 Gy, ‘Orito’ from 61.8 to 68.3 Gy, and ‘FHIA-01’ from 73.2 to 80.9 Gy. Fig. 11 shows

the optimum range for gamma rays irradiation in each cultivar. LD_{50} of the cultivars are indicated in bold squares.

In *Musa* cultivars significant differences were reported by Novak *et al*. (1990) in radio-sensitivity and post-irradiation recovery, assessed as an increase fresh weight, after irradiation of excised shoot-tips. The diploid line 'SH-3142' (AA), was the most sensitive to gamma irradiation, while the tetraploid 'SH-3436' (AAAA) expressed the lowest level of radiation damage among the seven clones tested. In the present study, the acute irradiation (^{60}Co) applied in a "Gamma Room" onto banana explants in Petri dishes, shows that all cultivars were affected by gamma doses. The survival ability decreased with increase in dosage, resulting in different rates dependent on varieties. 'Williams' showed good height, weight and survival rate even when the dosage reached 200, and 'FHIA-01' tolerated dosages of up to 500 Gy, but with low survival rate. On the other hand, 'Cavendish Enano' appeared to be more sensitive to gamma rays exposure, based on low plant height and weight recovered.

When the relationship between LD_{50} and ploidy group of the cultivars were compared, triploid cultivars showed higher values than the diploid and tetraploid cultivars. The results obtained in the present study are in contrast with the findings of Novak *et al*. (1990) who reported that these differences were dependent on the ploidy level and the hybrid constitution by genomes. Probably, the number of explants that was used at the present study was a few (20 per dish) in comparison to Novak *et al*. (1990) who used 200 explants for each cultivar. Van Harten (1998) in his book about mutation breeding mentioned that Novak and his associates discussed gamma irradiation of *in vitro* cultured shoot-tips of diploid, triploid and tetraploid cultivars with different genome of banana and plantain. Radio-sensitivity was assessed by determining fresh weight of cultures and the degree of shoot differentiation. Out of seven clones tested

a diploid clone (genomic constitution: AA) was not sensitive to the irradiation; a tetraploid clone (AAAA) showed the lowest level of irradiation damage. For diploid clones doses of 20-25 Gy are advised, for triploid clones (AAA, AAB, ABB) doses of 35-40 Gy and for tetraploid (AAAA) doses of 50 Gy.

3. 3. 2. Chronic irradiation (^{60}Co) at the "Gamma Field" in banana plants

Irradiated plants from "Gamma Field" were planted on *in vitro* culture to initiate multiplication as described in Fig. 12, where propagation of the material after irradiation is shown. Normal growth was observed in plants during the irradiation exposure for 34 days kept at "Gamma Field". After three propagations, 120 plants were obtained and screened to detect resistant/tolerant mutants to black Sigatoka at early stages using juglone as a host-specific toxin.

3. 3. 3. Chronic irradiation (^{137}Cs) at the "Gamma Greenhouse" in banana plants

At the "Gamma Greenhouse", plants from each cultivar kept for nine months showed observable unique characteristics, which demonstrate that damage could occur in the meristematic cells. Characteristics such as double leaf (DL), long leaf (LL), abnormal leaf (AL), rudimentary leaf (RL), right side short leaf (RSSL), spindled leaf (SL), and yellow spotted leaf (YSL) and normal leaf (NL) were recorded (Fig. 13). Table 4 summarizes the unique characteristics observed in the four cultivars. YSL was the most frequent observed characteristic in 'Cavendish Enano', 'FHIA-01' and 'Orito', except in 'Williams'.

Novak *et al*. (1990) reported considerable phenotypic variation among the plants regenerated from shoot-tips after mutagenic treatment. In early stages of plant development the irradiation affected emergence and expansion of the younger leaves and several plants formed compact leaf rosettes. Aberrant morphology of laminae was observed mainly in younger leaves which indicate a damage of the apical meristem.

The results from the present study at the "Gamma Greenhouse" coincided with his report when meristems of irradiated banana plant were found atrophied by high irradiation doses during preparations for *in vitro* propagation as is shown in Fig. 14. Yu (2006) also mentioned that young tissue with exuberant metabolism and fast growth has higher radiation sensitivity than more slowly growing adult and old tissue. Tissues that have fast cell division are more sensitive than tissues with slowly dividing cells; thus, the bud and roots of highly evolved plants are more sensitive than stem and leaves. In this study, in order to reduce such damage, short-term irradiation exposures could be explored. Double leaf and spindle were the most relevant characteristics. YSL was the most frequently observed characteristic in 'Cavendish Enano', 'FHIA-01' and 'Orito', except in 'Williams'.

3. 3. 4. Juglone toxin screening

An experiment carried out by Stierle *et al*. (1991) determined that juglone was the most biologically active phytotoxin which induced the formation of necrotic lesions on cultivars of banana at 0.1 µg. In the present study, when the three susceptible irradiated cultivars were tested using 150 ppm of juglone, the reaction within and across cultivars were different. Values ranging from 5.97 mm^2 to 45 mm^2 of necrotic area were observed. In 'Cavendish Enano' (94% of the evaluated plants) and

‘Williams’ (93%), the necrotic area values ranged from 15 mm^2 to 30 mm^2, except in Orito (95% of the evaluated plants) that varied from 10 mm^2 to 30 mm^2 (Fig. 15-A, -B and -C). A few plants among the three susceptible irradiated cultivars were observed showing a low value of necrotic area ranging from 5.97 mm^2 to 9.92 mm^2. The values in these plants were lower than the resistance indicator value (10.79±2.13 mm^2) which was selected in this study (Table 5).

Different screening methods have been mentioned by different authors (Harelimana *et al*., 1997; Molina and Krausz, 1988; Lepoivre *et al*., 2002). The leaf puncture bioassay has been widely used to assess host-tolerance/resistance to black Sigatoka or juglone; however, detached banana leaves or the injection of crude toxic extract into the leaves is easy but neither sensitive nor quantitative.

Clear variation between a black Sigatoka-susceptible cultivar ‘Grand Naine’ and the line ‘IV-9’ reported as highly resistant were observed using the needle-piercing method with a phytotoxic extract to measure the diameter of the area of necrotic tissue (Molina and Krausz, 1988). The toxic activity of the extracts apparently can be used to screen banana and plantain breeding materials rapidly even at very young stage of plant growth. This should prove very useful to hasten the resistance screening process (Molina and Krausz, 1988).

In the present study, irradiated plants from “Gamma Room” and “Gamma Field” were screened. 208, 179 and 307 plants were screened in ‘Cavendish Enano’, ‘Williams’ and ‘Orito’, respectively. Plants containing low values (5-10 mm^2 of necrosis produced by using 150 ppm of juglone on the leaf discs) were selected. 5, 8 and 20 plants were selected in ‘Cavendish Enano’, ‘Williams’ and ‘Orito’, respectively. In studies mentioned by FAO/IAEA (2002) it was also found that from around 4,000 irradiated ‘Grande Naine’ plants screened, 19 putative mutants were

selected for their tolerance to 25 ppm of Juglone, but new screening for resistance to *M. fijiensis* in these plants still has to be confirmed through inoculation with the fungus, which is a very slow process. On the other hand, the results obtained by Lepoivre *et al*. (2002) confirm the possibility of selecting banana plants resistant to *M. fijiensis* metabolites but in their research this approach did not result in higher resistance to black leaf streak disease.

Since the leaf discs of the resistant cultivar (in this case 'FHIA-01') were less affected by juglone (a host-specific toxin) applied at 150 ppm, in contrast to susceptible cultivars, the leaf disc immersion method can be a simple and rapid method to measure the reaction for tolerance/resistance to juglone. A low extent of necrosis in susceptible irradiated cultivars was observed (selected ones); however, new studies to reconfirm tolerance/resistance to juglone on the selected material must be undertaken. Van Harten (1998) mentioned that in the case of selection with phytotoxins and culture filtrate appears to be more effective than the use of the pathogen itself.

Lepoivre *et al*. (2002) reported that chloroplast is target site of juglone. When juglone was used, swelling chloroplasts were observed by electron microscopy in ethyl acetate crude extract (EaCE)-treated leaves. Upon observation, 'Fougamou' (a partially resistant cultivar) chloroplasts appeared to be less affected by juglone in contrast to 'Grand Naine'. These results suggest that the chloroplast is one of the primary action sites of juglone.

3. 3. 5. Relative DNA content measuring by using flow cytometer

Histograms of the relative DNA content of 'Cavendish Enano', 'Williams' and 'FHIA-01' are showing in Fig. 16. The relationships

between the irradiation doses and the relative DNA content (Fig. 17) showed that the values from the three cultivars ('Cavendish Enano', 'Williams' and 'Orito') showed an imperceptible variation in all the applied doses. However, a perceptible variation was possible to observe in 'FHIA-01' when higher doses were applied. The variation of 'FHIA-01' was better observed when a frequency distribution of the relative DNA contents was analyzed (Fig. 18). The analysis detected reduction of the relative DNA content when the irradiation dose was increased. The lowest value was 1.947 (96% of donor), observed at 300 Gy. These results suggest that deletion of DNA was occurred when high doses were applied in 'FHIA-01', exhibiting a shift to the left, indicating loss of whole or part of chromosomes. Aneuploid is the mean having one or more complete chromosomes in excess of, or less than the normal haploid, diploid or polyploidy number characteristics of the species. Seventeen plants with lower DNA content were selected corresponding to the doses of 200 and 300 Gy.

FAO/IAEA (2002) screened aneuploid mutants by flow cytometric analysis in irradiated banana plants. Aneupliod mutants can be detected through chromosome counts with rather time-consuming process. Results obtained by flow-citometry were compared to chromosome counting in meristem shoot-tip cells. It could be shown that flow cytometry is sensitive enough to detect aneuploidy in bananas. With such a sensitive and fast technique we are now screening routinely for aneuploid mutants in addition to screening for juglone resistance. Aneuploid mutants would be important tools for basic research in *Musa.*

Dolezel *et al*. (2002) reported that flow cytometry have been used to determine ploidy levels of *Musa* accessions. Flow cytometric ploidy assay involved preparation of suspensions of intact nuclei from small amount of the leaf tissue and the analysis of fluorescence intensity

after staining with DAPI. Chicken red blood cell (CRBC) nuclei were included in all samples as an internal reference standard. Out of 890 accessions 2% of them were detected showing mixed ploidy. A reliable and high-throughput system for ploidy screening in *musa* is important outcome of the study. The use of the CRBC nuclei, allowed high-resolution analysis, and the results obtained so far indicated suitability of this system for rapid detection of aneuploid.

In the annual report published by National Institute of Agrobiological Sciences (2002), the relationship between specific hairless mutants of sugarcane which lacked 57 groups hair and their nuclear DNA contents was investigated. As results, the rates of hairless mutation obviously increased as the irradiation doses. A very high mutation rate was observed at 200 Gy. All the hairless mutants lacked more than 1.4% of the DNA compared with the donor. DNA deletions frequently occurred at more than 100 Gy.

3. 3. 6. Putative mutant of FHIA-01

Two putative mutants of ‘FHIA-01’ were observed during the development of the selected plants (17 plants with lower DNA content) in greenhouse conditions. One of them showed dwarfism, but unfortunately was died during the development stage and another plant shows dropping leaves. Unfortunately, in January, 2006 on winter time the plants were damaged by chilling injury, most of them with bunch (Fig. 19). Fig. 20 shows the height and circumference of the mother plants and suckers before chilling injury occurred. In the figure, the plants with the code numbers ‘F II 300 6-20’ and ‘F I 200 4-4’ show fast growth of suckers, which is an important characteristic for earliness crop cycle. Mother plants were cut to recover the suckers. Suckers were recovered well and at the

moment they are at the development stage. Sucker from the mother plant which showed drooping leaves also shows the same characteristic, compared with the normal growth showed by the others selected plants (Fig. 21). This characteristic, remarkable in the sucker, confirm that the putative traits are transmissible to the next generation. Taken together, gene has been expressed and highly heritance will occur in the progeny. The cultivar 'FHIA-03', tetraploid (AABB) showed also the same pattern of leaves showing attractiveness during its developing period having big bunches and high production (Reyes-Borja, 1995).

3. 3. 7. Factor of effectiveness produced by gamma rays

The factor of effectiveness was calculated using the amount of plants with variations (Table 6, 7, 8 and 9). 'Cavendish Enano', 'Williams' and 'Orito' showed a factor of effectiveness of 7.49 in selected plants tolerant to juglone. Other useful characteristics such as low DNA content, dwarfism, sigmoid drooping leaf, fast growth sucker were also observed. Leaves abnormalities were most observed when the plants were subjected to long chronic irradiation period under greenhouse condition.

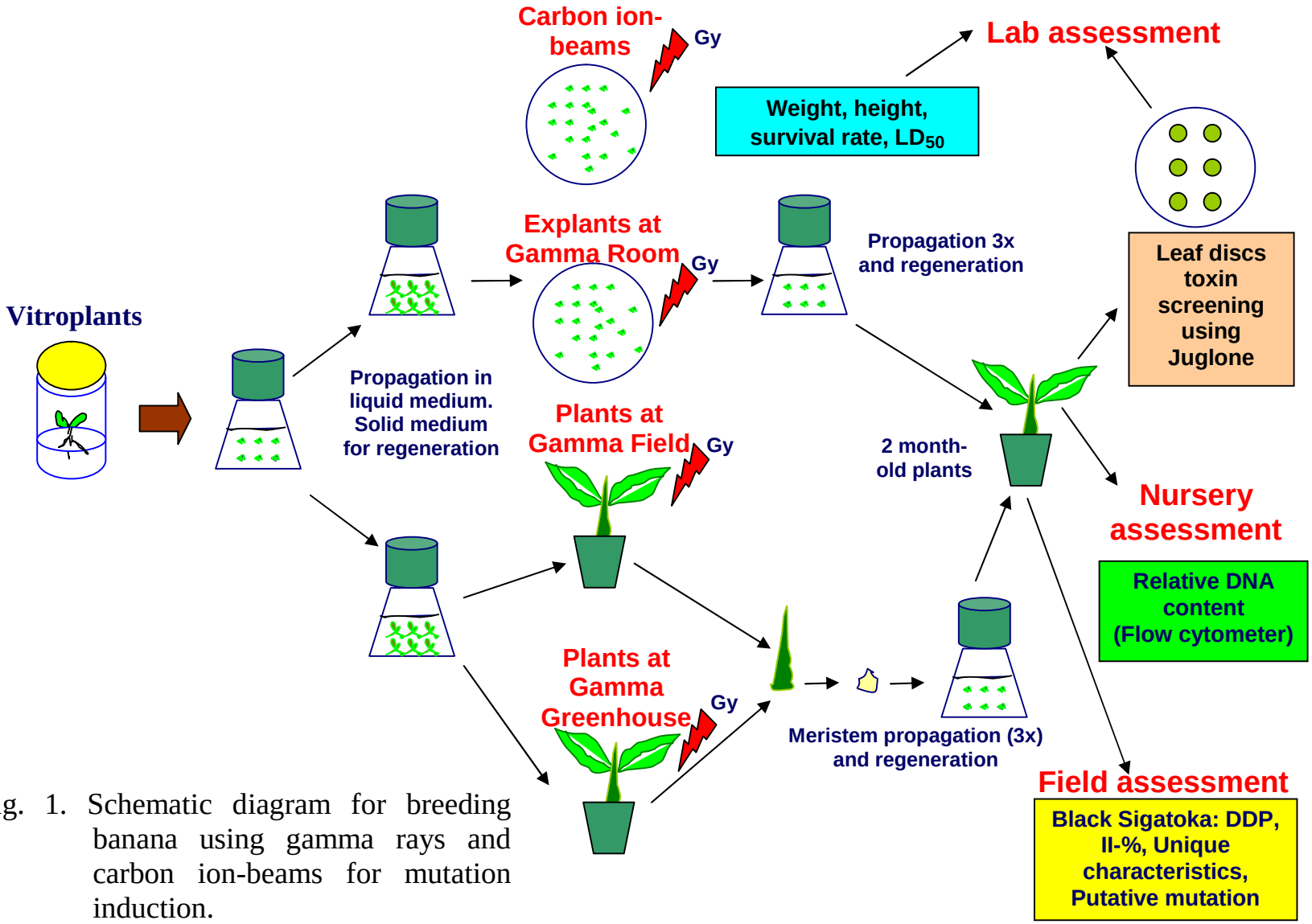

Fig. 1. Schematic diagram for breeding banana using gamma rays and carbon ion-beams for mutation induction.

Fig. 2. Cultivars of banana used in the experiments. ‘Cavendish Enano’ (A), ‘Orito’ (B), ‘FHIA-01’ (C) and ‘Williams’ (D).

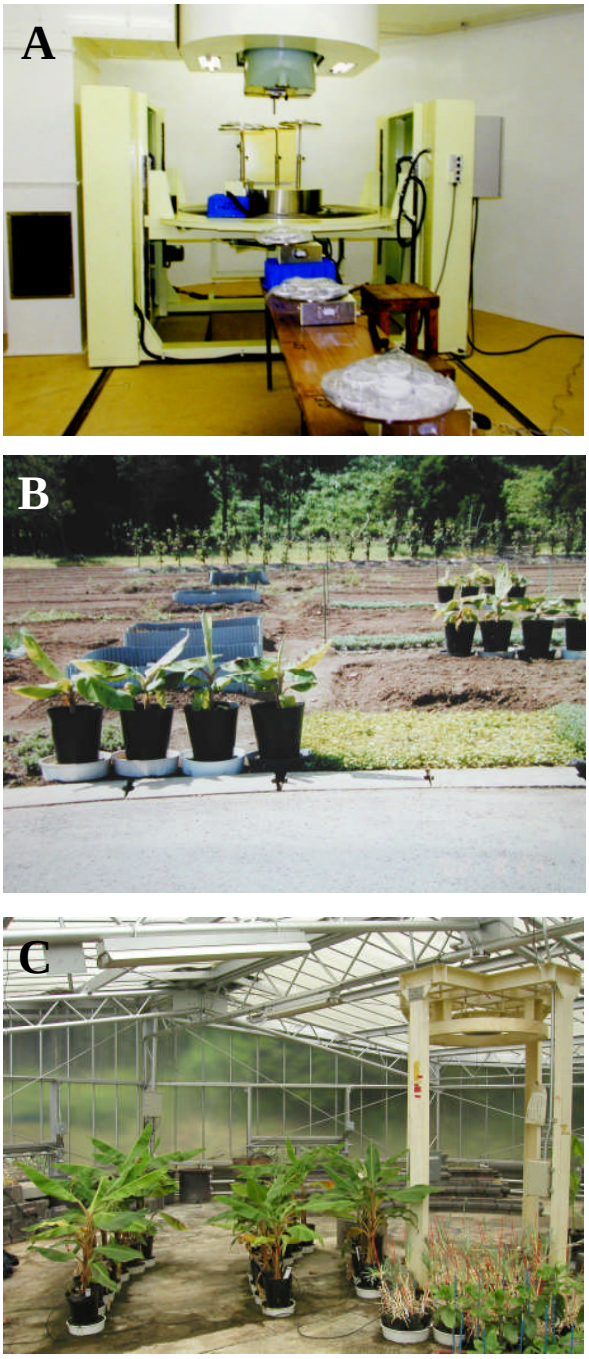

Fig. 3. Banana explants in dishes 90 mm at “Gamma Room” (A). Banana plants at the “Gamma Field” (B) and “Gamma Greehouse” (C). Dosage depended about the distance from the ^{60}Co source where the explants or plants were located.

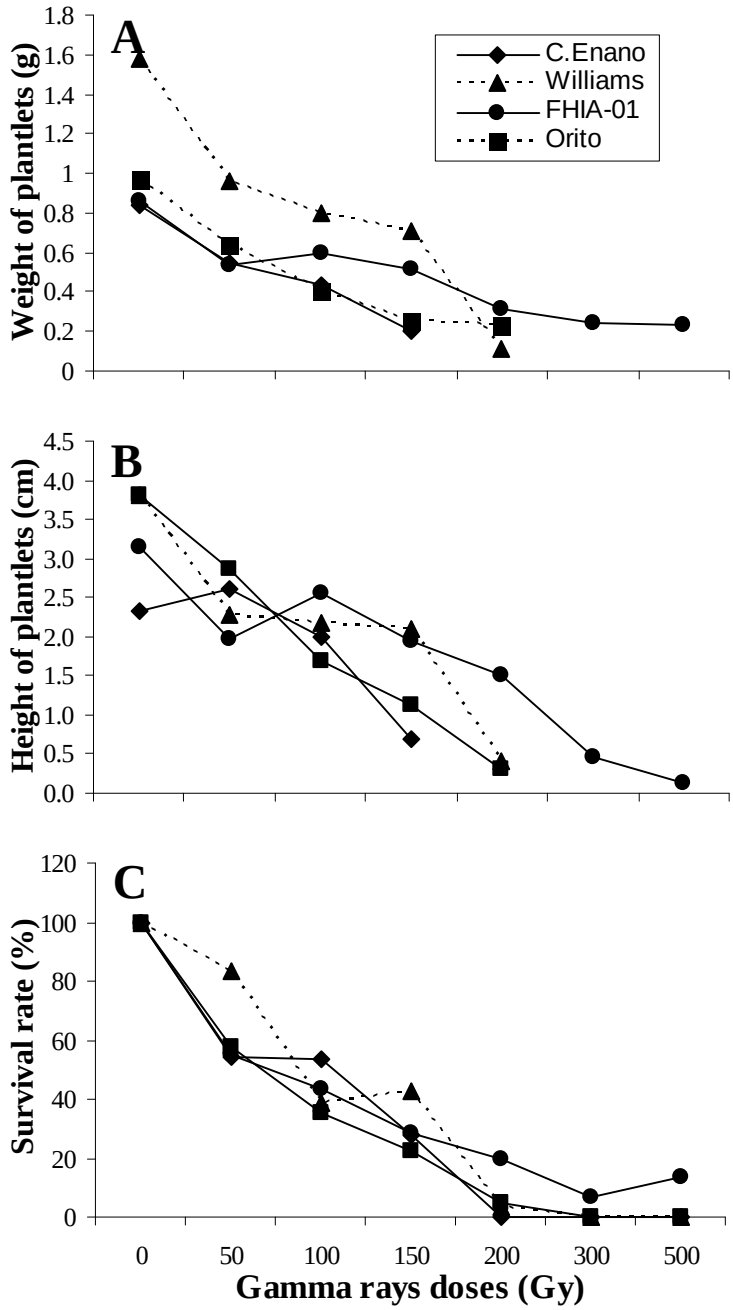

Fig. 4. Weight (A), height (B) and survival rate (C) of the plantlets one month after irradiation with different dosage in four cultivars of banana.

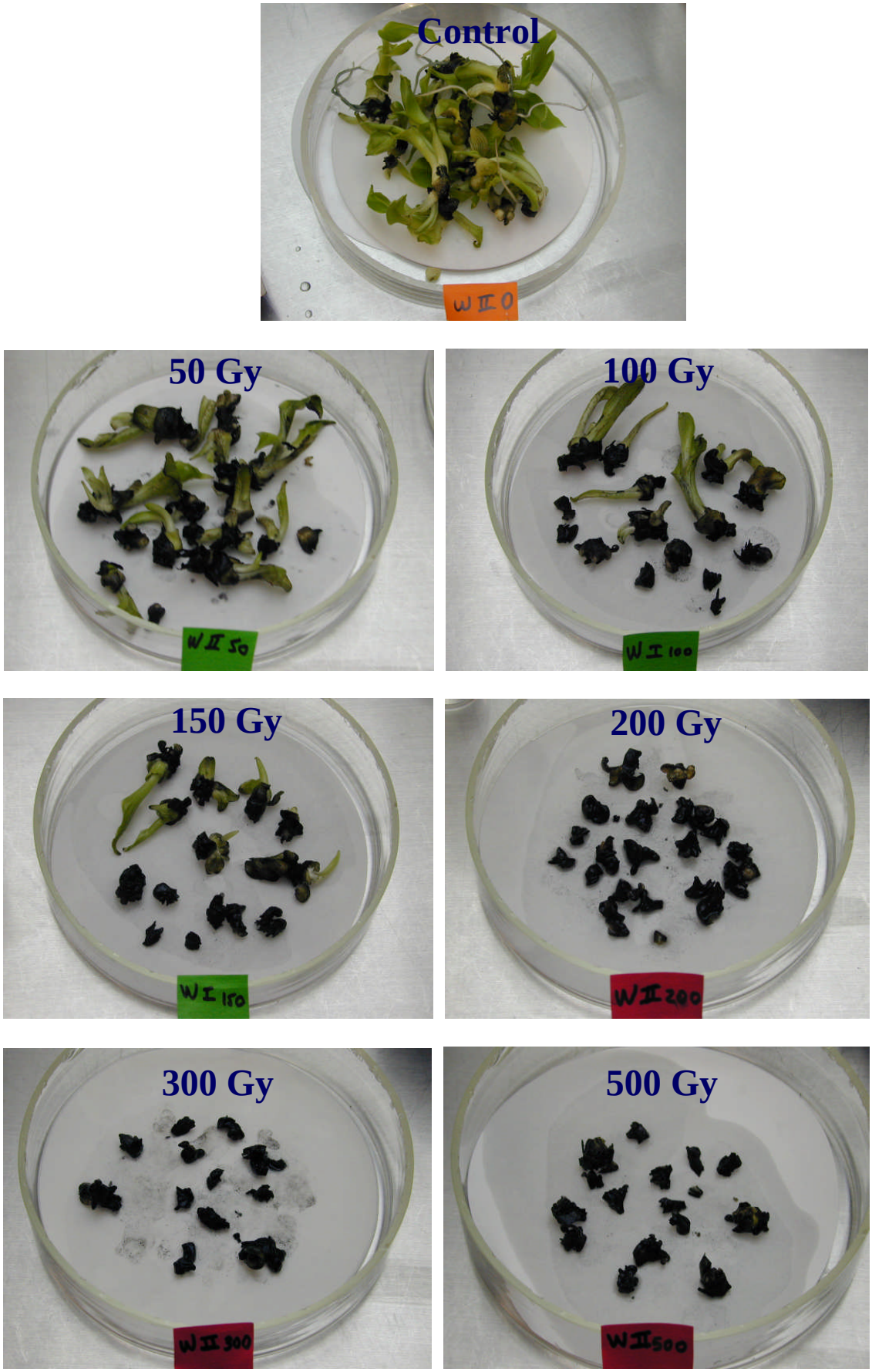

Fig. 5. The photographs showing the effectiveness of irradiation doses in banana explants of 'Williams'.

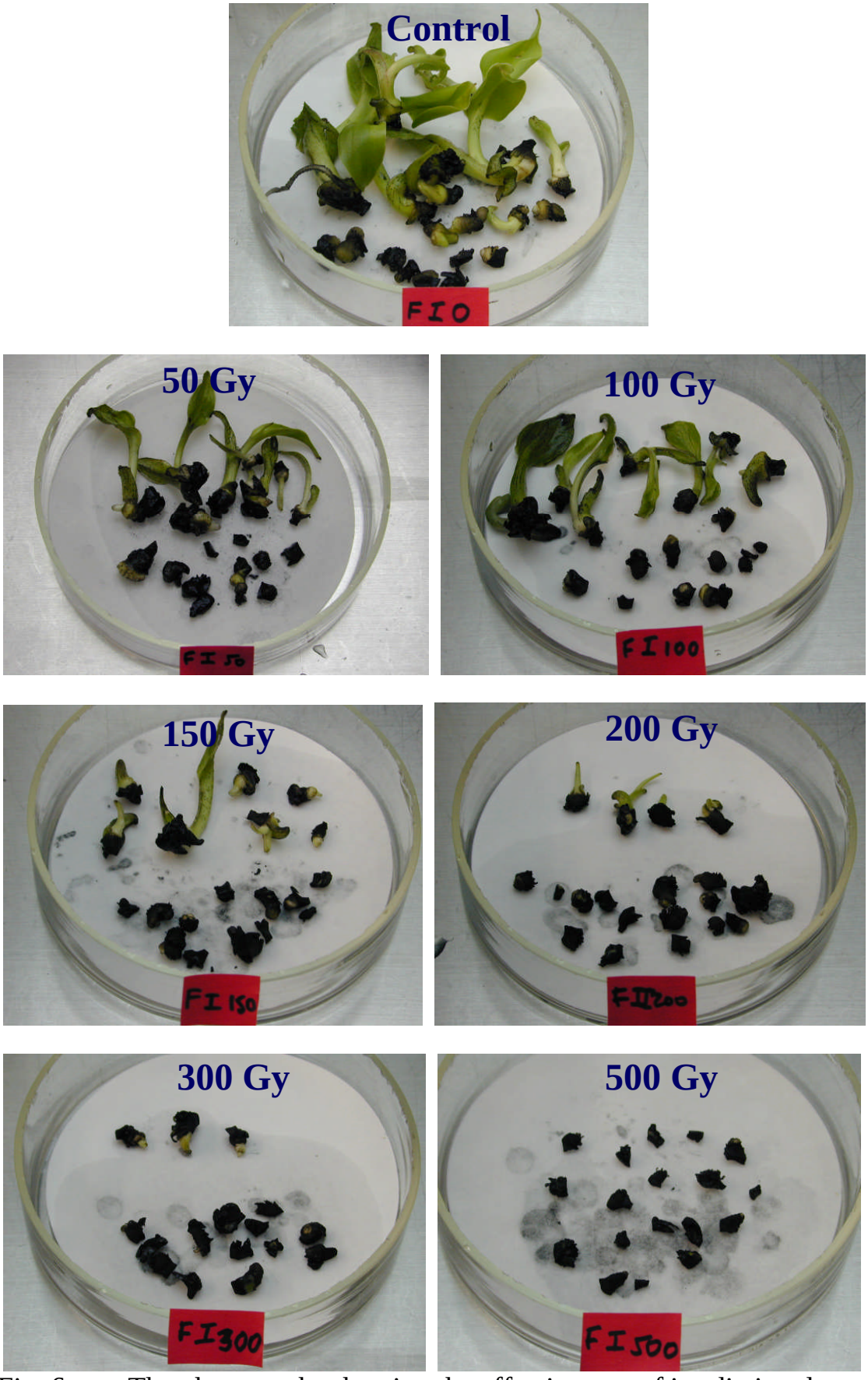

Fig. 6. The photographs showing the effectiveness of irradiation dose in banana explants of 'FHIA-01'.

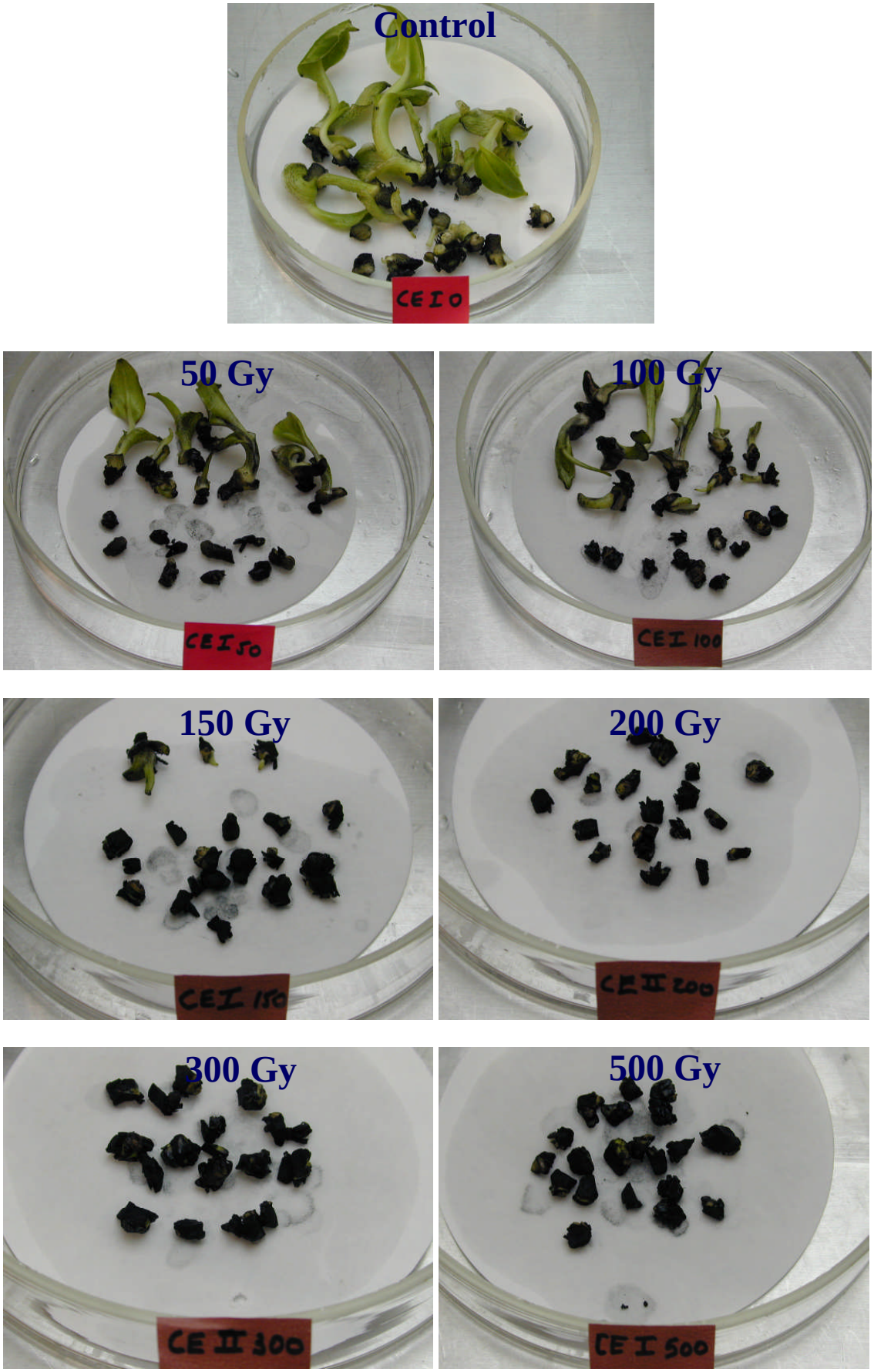

Fig. 7. The photographs showing the effectiveness of irradiation dose in banana explants of 'Cavendish Enano'.

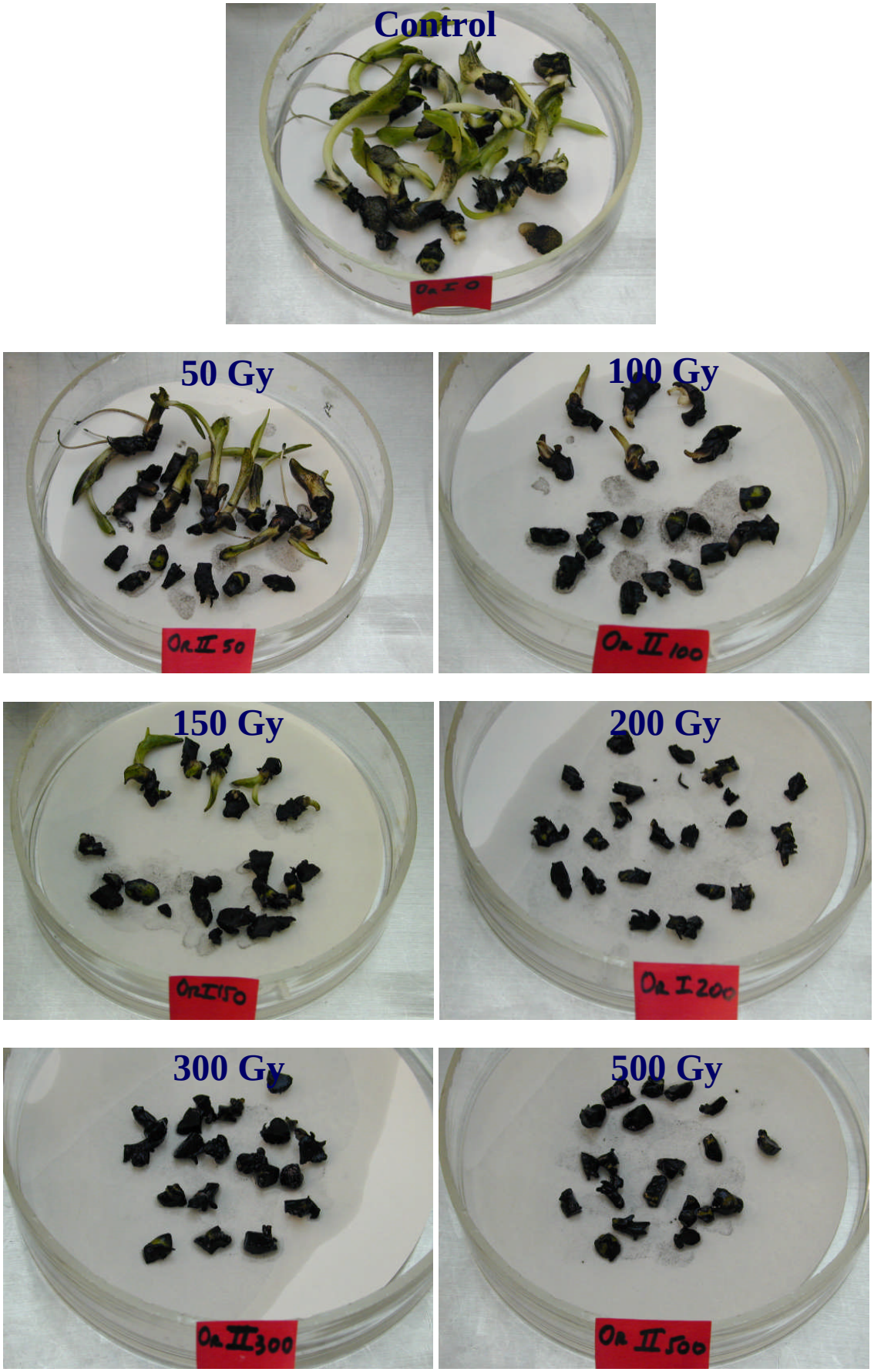

Fig. 8. The photographs showing the effectiveness of irradiation dose in banana explants of ‘Orito’.

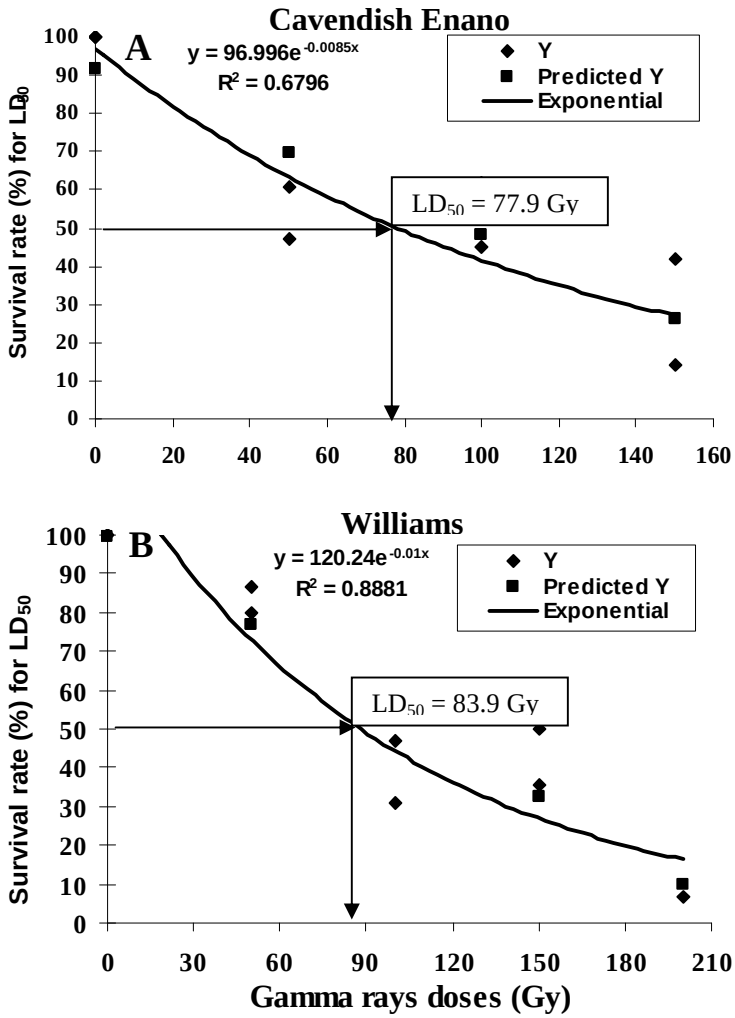

Fig. 9. Relationships between survival rate (%) for LD_{50} and gamma ray doses for LD_{50} in 'Cavendish Enano' (A) and 'Williams' (B).

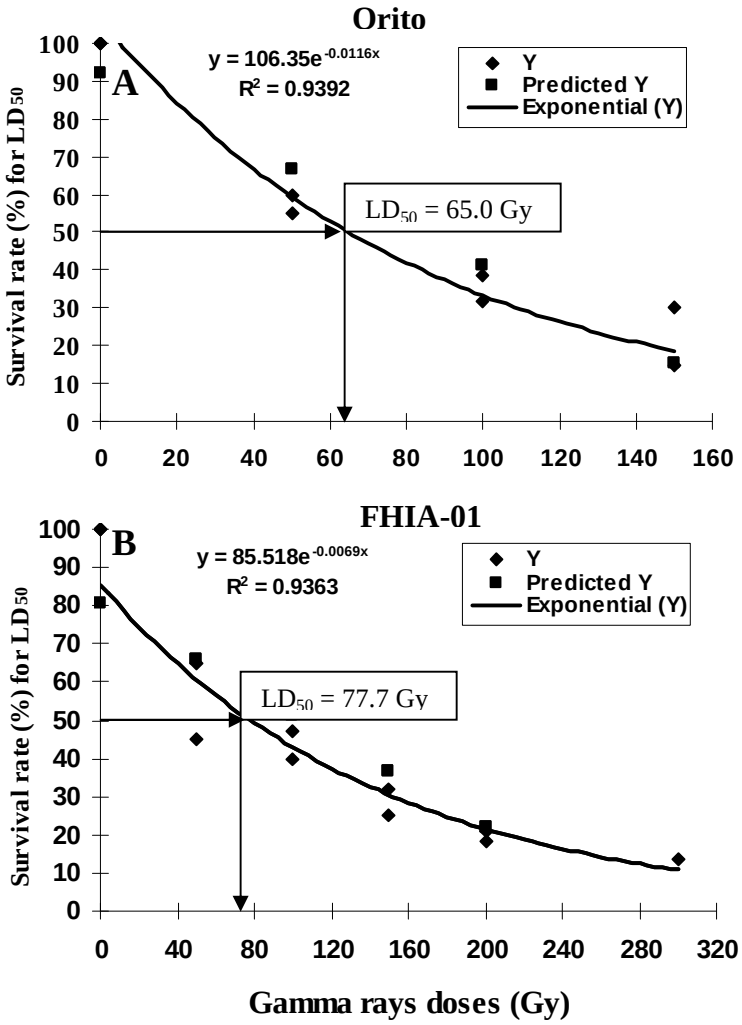

Fig. 10. Relationships between survival rate (%) for LD_{50} and gamma ray doses for LD_{50} in 'Orito' (A) and 'FHIA-01' (B).

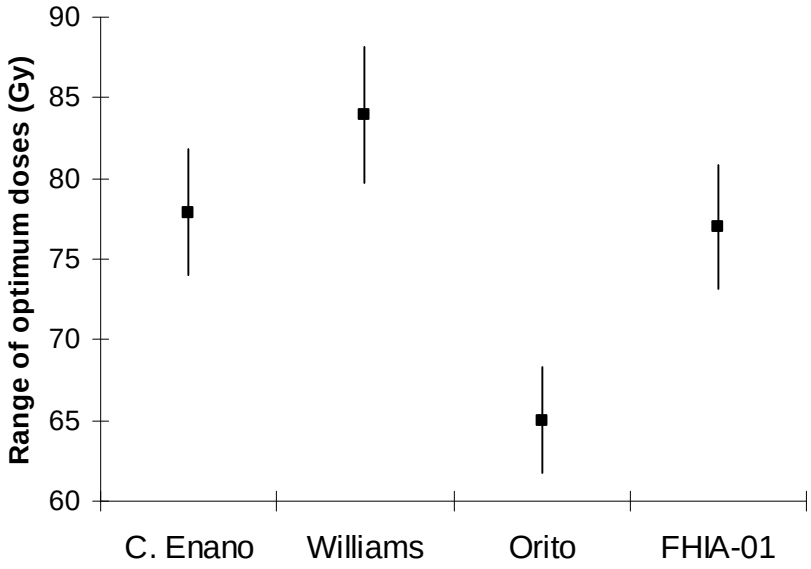

Fig. 11. Optimum range of gamma ray doses (Gy) obtained by plus/minus (±) 5% of the LD_{50} values. Bold squares represent the LD_{50} values in four cultivars of banana.

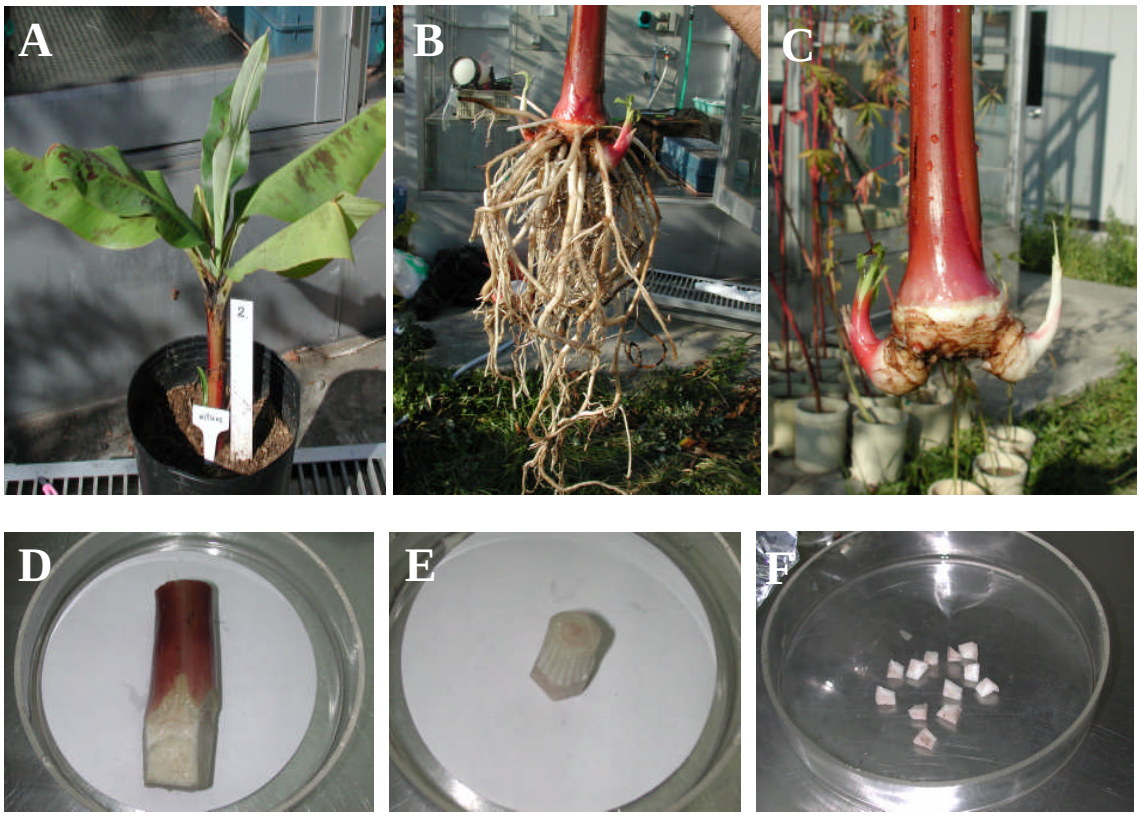

Fig. 12. A plant from “Gamma Field” 34 days after irradiation (A). Process of obtaining explants and placing into Petri dish (B, C, D and E). Petri dish containing explants ready for putting into an initiation solid medium (F).

Fig. 13. Unique characteristics observed at the “Gamma Greenhouse” irradiated plants after 9 months. A, abnormal leaf (AL); B, double leaf (DL); C, rudimentary leaf (RL); D, spindled leaf (SL); E, yellow spotted leaf (YSL) and F, long leaf (LL).

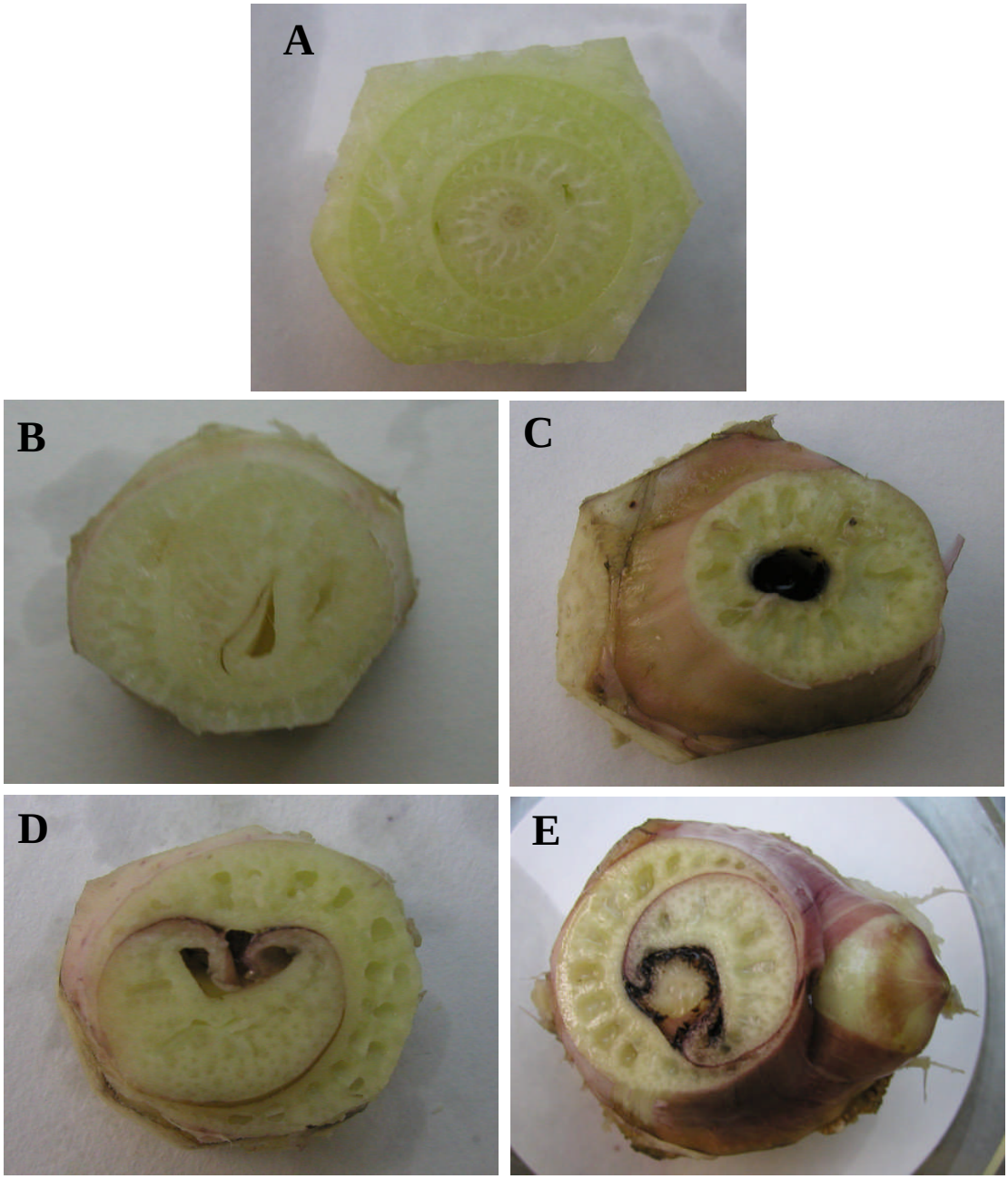

Fig 14. Banana meristems affected by long period-chronic irradiation (^{137}Cs) applied in the "Gamma Greenhouse". 'Cavendish Enano' (A, Normal meristem-0 Gy). 'Cavendish Enano' 0.50 Gy (B and C), 'FHIA-01' 0.50 (D and E).

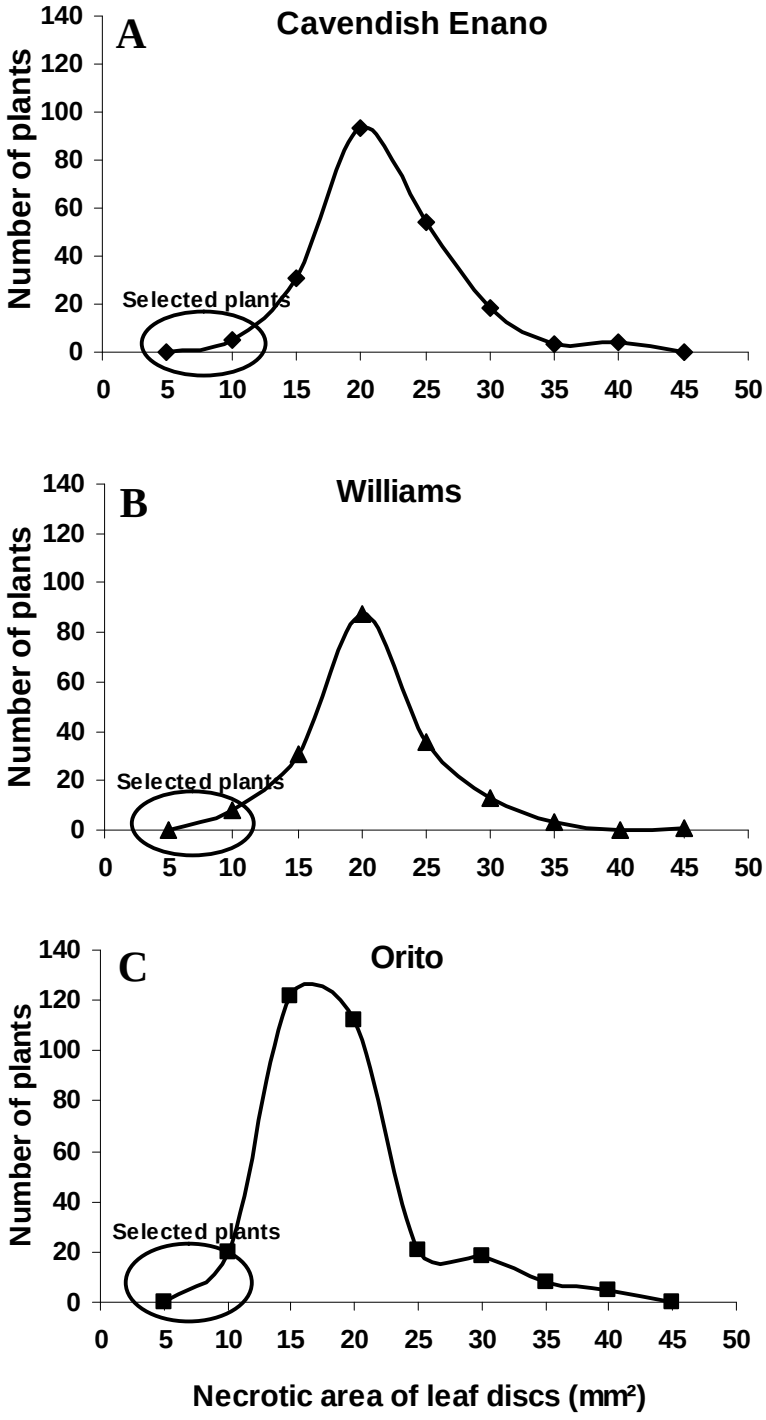

Fig. 15. Frequency distributions of necrotic area of leaf discs in irradiated populations of the banana cultivars. 'Cavendish Enano' (A, 208 plants), 'Williams' (B, 179 plants) and 'Orito' (C, 307 plants).

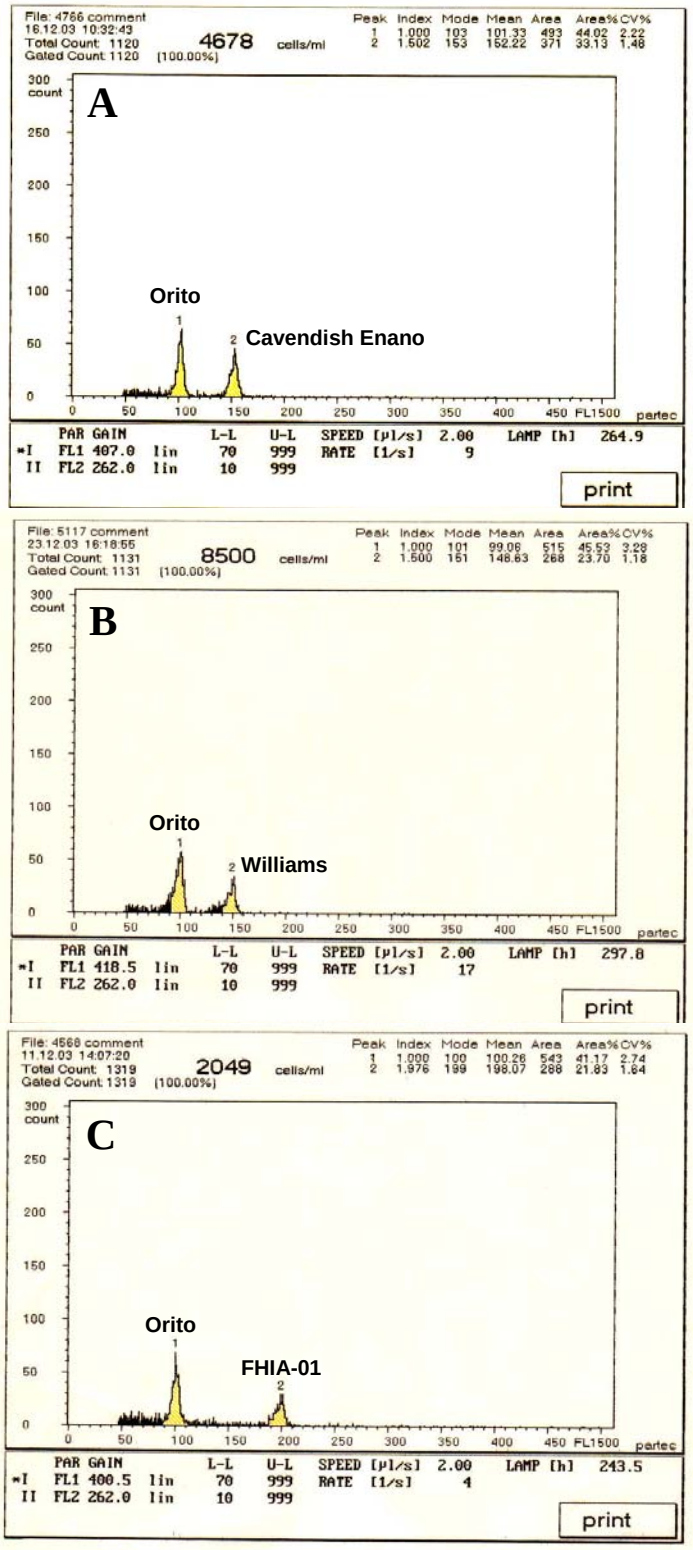

Fig. 16. Histograms of relative DNA content obtained during ploidy screening by flow cytometry for non irradiated banana. Pick 1 represent the cultivar 'Orito' used as an internal reference standard (A, B, and C) and # 2 pick is the analyzed cultivar:'Cavendish Enano'(A),'Williams' (B) and 'FHIA-01' (C).

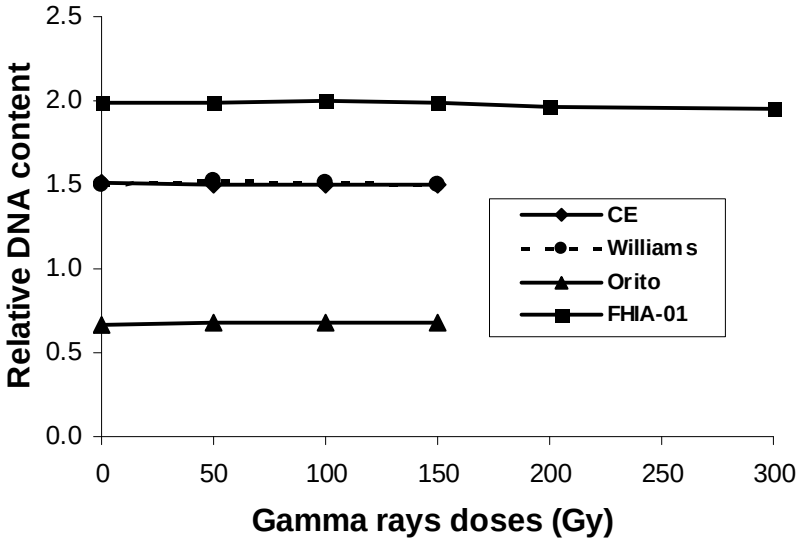

Fig. 17. Relationships between relative DNA contents and doses of irradiation in four cultivars of banana.

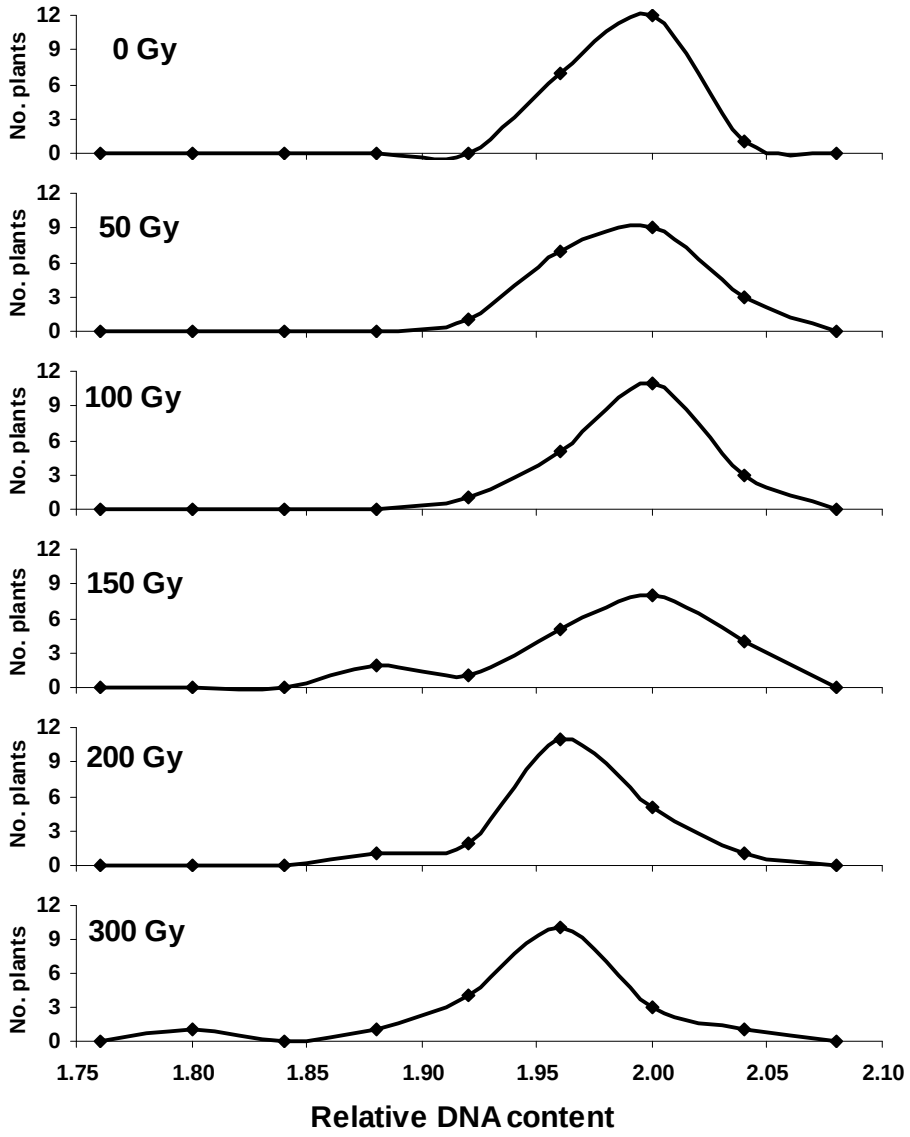

Fig. 18. Frequency distributions of the relative DNA contents in a population of 'FHIA-01' with different Gamma ray doses.

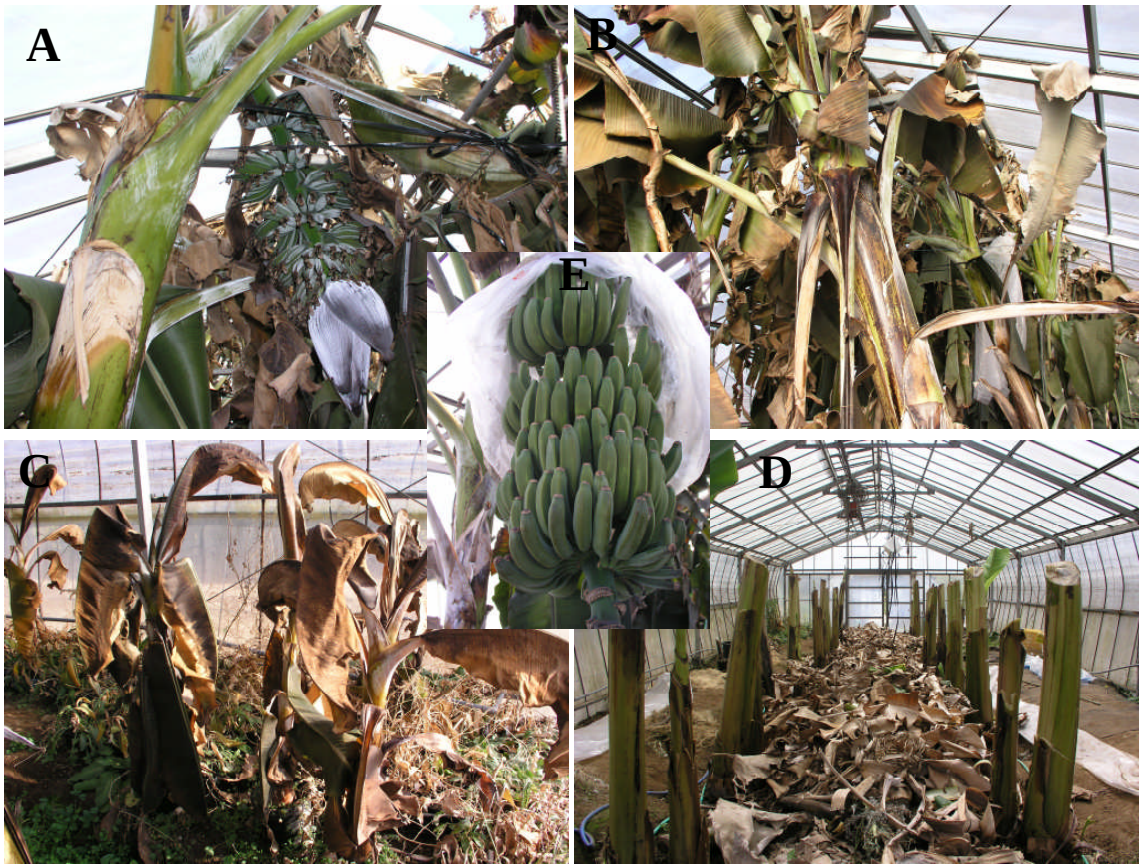

Fig. 19. Chilling injury plants of the ‘FHIA-01’ (selected population) at the greenhouse conditions (A-D). Injured plant on bunch development stage (A). Leaves injure plant (B). Dwarf plant died by chilled (C). Injured bunch used be at the development stage (E). Mother plants cut to recovering suckers (D).

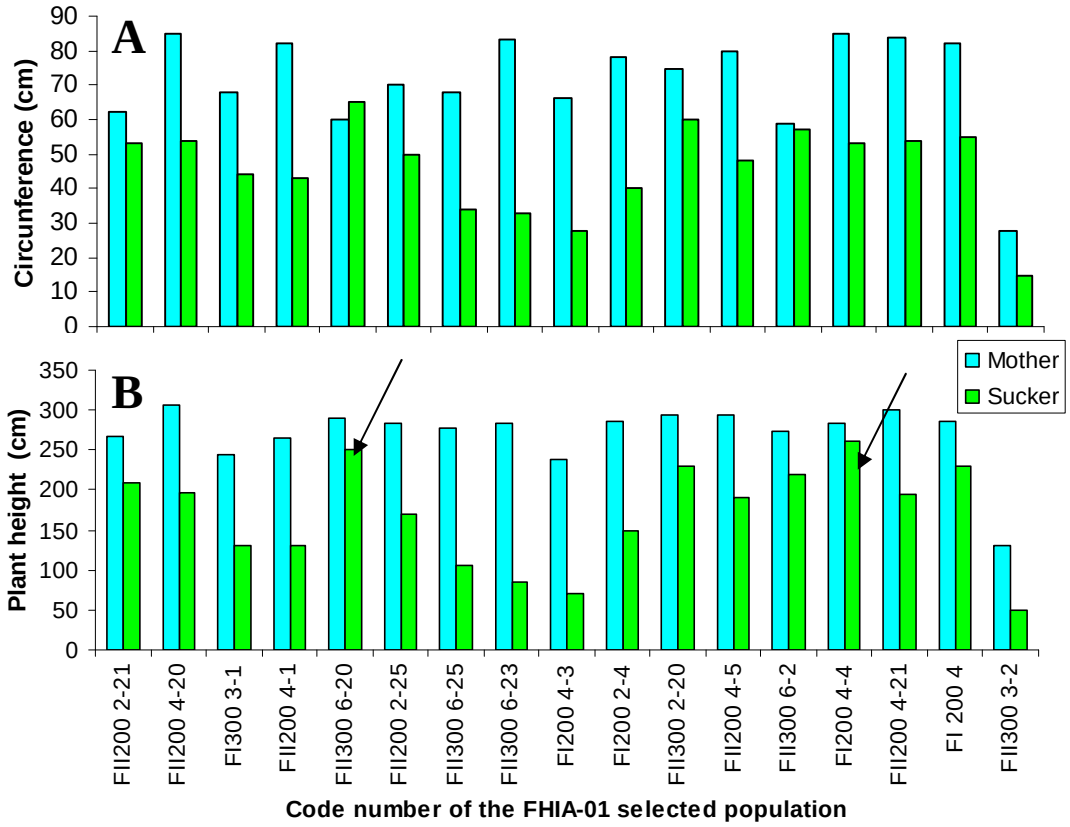

Fig. 20. Mother plant and sucker height (A) and circumference (B) of the pseudostem during chilling injure at the greenhouse condition. Arrows shows fast growth of suckers.

Fig.21. FHIA-01 mutant plant (A) and its petioles (B). Norman leaves shape of FHIA-01 and petioles (C-D), growing at greenhouse conditions (October 10, 2006).

Table 1. Differences of the plantlet weight (g) one month after irradiation by different dosage in four cultivars of banana at “Gamma Room”.

Dosage (Gy)	Cultivars of banana			
	C. Enano	Williams	FHIA-01	Orito
0	0.84a	1.58a	0.86a	0.97a
50	0.55ab	0.96 b	0.54abc	0.63ab
100	0.44abc	0.80 b	0.60ab	0.40 bc
150	0.20bc	0.71 b	0.52abc	0.25 bc
200	0.00c	0.12 c	0.31 bc	0.23 bc
300	0.00c	0.00 c	0.24 bc	0.00 c
500	0.00c	0.00 c	0.23 c	0.00 c
F. value	**11.76**	**45.54**	**9.32**	**13.12**
Significance	**	**	**	**

** Significant (P≦0.01).

Numbers in the same column followed by a different letter are significant (P≦0.01) level according to Tukey-Kramer analysis.

Table 2. Differences of the plantlet height (cm) one month after irradiation by different dosage in four cultivars of banana at "Gamma Room".

Dosage (Gy)	Cultivars of banana			
	C. Enano	Williams	FHIA-01	Orito
0	2.32a	3.80a	3.13a	3.82a
50	2.60a	2.26ab	1.98ab	2.86ab
100	1.99ab	2.16ab	2.56ab	1.69 bc
150	0.70bc	2.09ab	1.95ab	1.13 bc
200	0.00c	0.40 bc	1.51ab	0.30 c
300	0.00c	0.00 c	0.45 b	0.00 c
500	0.00c	0.00 c	0.14 b	0.00 c
F. value	**6.35**	**15.79**	**6.18**	**19.32**
Significance	*	**	*	**

*, ** Significant ($P \leqq 0.05$) and ($P \leqq 0.01$), respectively.

Numbers in the same column followed by a different letter are significant ($P \leqq 0.05$) level according to Tukey-Kramer analysis.

Table 3. Differences of the survival rate (%) one month after irradiation by different dosage in four cultivars of banana at “Gamma Room”.

Dosage (Gy)	Cultivars of banana			
	C. Enano	Williams	FHIA-01	Orito
0	100.0a	100.0a	100.0a	100.0 a
50	54.1a	83.3a	55.0ab	57.5a
100	53.5a	38.7 b	43.5abc	35.2 b
150	28.0ab	42.9 b	28.4abc	22.5 bc
200	0.0 b	3.3 c	19.6 bc	5.0 cd
300	0.0 b	0.0 c	6.8 bc	0.0 d
500	0.0 b	0.0 c	13.9 c	0.0 d
F. value	**13.21**	**85.57**	**8.57**	**50.1**
Significance	**	**	**	**

** Significant (P≦0.01).

Numbers in the same column followed by a different letter are significant (P≦0.05) level according to Tukey-Kramer analysis.

Table 4. Unique characteristics observed in four cultivars of banana irradiated during nine months at "Gamma Greenhouse".

Cultivars	Total plants	Characteristics								Total leaf/total plants
		AL*	DL	LL	RL	RSSL	SL	YSL	NL	
Orito	**5**	3	0	13	0	1	1	17	29	64
C.Enano	**5**	5	1	9	1	0	1	8	42	67
Williams	**3**	10	0	0	0	1	0	0	21	33
FHIA-01	**5**	0	0	2	0	0	0	26	45	73

*Abbreviations: Abnormal leaf (AL), double leaf (DL), long leaf (LL), rudimentary leaf (RL), right side short leaf (RSSL), spindled leaf (SL), yellow spotted leaf (YSL) and normal leaf (NL).

Table 5. Selected materials reporting less than 10 mm^2 of the leaf discs necrotic area affected by 150 ppm of juglone solution.

No.	Orito		Williams		Cavendish Enano	
	Code No.	mm^2	Code No.	mm^2	Code No.	mm^2
1	Or-I-50-4-165	9.01	W-II-50-12-29	7.85	CE-I-100-7-1	9.45
2	Or-I-50-5-141	8.49	W-II-50-2-64	8.94	CE-II-100-10-92	6.69
3	Or-II-50-4-220	6.83	W-I-150-1-12	7.93	CE-II-100-11-97	7.25
4	Or-II-50-6-219	8.50	W-I-150-2-2	7.42	CE-II-150-4-94	7.13
5	Or-II-50-7-81	9.88	W-I-150-4-14	8.89	CE-2-11*	9.54
6	Or-II-50-7-105	7.98	W-I-150-5-9	9.84		
7	Or-II-50-8-117	5.97	W-I-150-6-5	6.11		
8	Or-II-50-11-206	8.29	W-0.5-2*	9.01		
9	Or-I-100-1-1	9.42				
10	Or-I-100-1-133	9.92				
11	Or-I-100-2-209	7.59				
12	Or-I-100-2-213	8.15				
13	Or-I-100-3-211	6.61				
14	Or-I-100-3-216	9.87				
15	Or-I-100-5-136	9.26				
16	Or-II-100-1-158	9.72				
17	Or-I-150-1-105	9.74				
18	Or-I-150-2-4	9.06				
19	Or-I-150-6-137	8.91				
20	Or-II-150-3-1	9.72				

*These plants are selections from “Gamma Field”, the rest are from “Gamma Room”.

Table 6. Factor of effectiveness and putative mutations by Gamma rays observed in 'Cavendish Enano'.

Mutagen/ facility	No. of total plants	No. plants with variation	Characteristics	Gy dose inducing mutation (*n*)	Factor of effectiveness (%)
Gamma ray/Room	282	4	Tolerant to juglone	100 (3) 150 (1)	1.63 0.35
Gamma ray/Field	26	1	Tolerant to juglone	2 (1)	3.84
Gamma ray/Greenhouse	5	5	AL AL, DL RL, LL, SL, YSL, LL YSL, LL	0.25 (1) 0.25 (1) 0.5 (1) 0.5 (1) 0.75 (1)	20.0 20.0 20.0 20.0 20.0

LL (long leaf), YSL (yellow spotted leaf), SL (spindled leaf), RL (rudimentary leaf), AL (abnormal leaf) and DL (double leaf).

Table 7. Factor of effectiveness and putative mutations by Gamma rays observed in 'Williams'.

Mutagen/ facility	No. of total plants	No. plants with variation	Characteristics	Gy dose inducing mutation (*n*)	Factor of effectiveness (%)
Gamma ray/Room	156	7	Tolerant to juglone	50 (2) 150 (5)	1.28 3.20
Gamma ray/Field	14	1	Tolerant to juglone	0.5 (1)	7.14
Gamma ray/Greenhouse	3	3	AL AL, RSSL AL	0.25 (1) 0.5 (1) 0.75 (1)	33.3 33.3 33.3

AL (abnormal leaf) and RSSL (right side short leaf)

Table 8. Factor of effectiveness and putative mutations by Gamma rays observed in 'Orito'.

Mutagens/ facility	No. of total plants	No. plants with variation	Characteristics	Gy dose inducing mutation (*n*)	Factor of effectiveness (%)
Gamma ray/Room	282	20	Tolerant to juglone	50 (8) 100 (8) 150 (4)	2.83 2.83 1.41
Gamma ray/Greenhouse	5	5	SSL, LL, YSL RSSL, AL LL, YSL LL, YSL, AL LL, YSL, AL	0.25 (1) 0.25 (1) 0.5 (1) 0.5 (1) 0.75 (1)	20.0 20.0 20.0 20.0 20.0

LL (long leaf), YSL (yellow spotted leaf), AL (abnormal leaf), SSL (spindled short leaf) and RSSL (right side short leaf).

Table 9. Factor of effectiveness and putative mutations by Gamma rays observed in 'FHIA-01'.

Mutagens/ facility	No. of total plants	No. plants with variation	Characteristics	Gy dose inducing mutation (*n*)	Factor of effectiveness (%)
Gamma ray/Room	120	18	Low DNA content	200 (10) 300 (7)	8.33 5.83
Gamma ray/Room	120	1	Dwarfism	300 (1)	0.83
Gamma ray/Room	120	1	Sigmoid drooping leaf	200 (1)	0.83
Gamma ray/Room	120	2	Fast growth sucker	200 (1) 300 (1)	0.83 0.83
Gamma ray/Greenhouse	5	5	YLS, LL YLS, LL YLS YLS YLS	0.25 (1) 0.25 (1) 0. 5 (1) 0. 5 (1) 0. 75 (1)	20.0 20.0 20.0 20.0 20.0

YSL (yellow spotted leaf) and LL (long leaf).

Chapter 4

Mutation induction on banana (*Musa* spp.) by using carbon ion-beams irradiation technique and identification of black Sigatoka resistant/tolerant mutants

4. 1. Introduction

Banana breeding programs around the world have been conducted for the enhancement of the new banana cultivars, but the low reproductive fertility and polyploidy levels make the traditional hybridization techniques remain difficult (Rowe, 1984). Many researches are aiming 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. It is an airborne fungal disease often diffuse in nature, producing large areas of black necrosis which become gray and the entire leaf can be infected and dies off (Craenen and Ortiz, 1996). Biotechnology and gene technology, together with conventional methods can assist to overcome these problems in developing new banana cultivars. Progress in the development of various biotechnologies has greatly contributed to the application of induced mutations in a wide range of plant species (Jain, 2001). FAO/IAEA (2006) officially reported more than 2500 (released) mutants obtained by different mutagenic inducing techniques. Among them, 1009 were obtained by applying gamma rays and 11 varieties were obtained by ion beam irradiation. The ion-beams technique has recently been used, which produce a wide number of mutants more effectively than gamma rays. Fukuda *et al*. (2003) mentioned that ion-beams can frequently produce large DNA alteration such as inversion, translocation, and large

deletion rather than point mutation, resulting in producing characteristics mutant induced by ion-beams. However, the characteristics of ion-beams on mutation induction have not been clearly elucidated yet.

Using the carnation variety 'Vital' Okamura *et al*. (2003) obtained a wide mutation spectrum in flower color and shapes compared with gamma rays application. Commercial cultivars were released in the spring 2002 in Japan from these mutants named as 'Red Vital Ion', 'Dark-Pink Vital Ion' and 'Misty Vital Ion', expecting an economic impact of about 10 billion yen.

Concerning to genetic improvement of banana, different techniques have been applied. Using gamma rays as mutagen, FAO/IAEA (2006) reported a banana mutant named 'Klue Hom Thong KU1' in Thailand, where the main improved characteristic was the bunch size and cylindrical shape. Roux (2001) mentioned that other desirable variants and putative mutants of banana have been identified for release or further confirmation trials in several countries. Cuba obtained the 'SH-3436-L9' and '6.44' mutants by gamma rays having a height reduction from the original plant. In Malaysia, both cultivars 'Mutiara' and 'Novaria' are tolerant to *Fusarium oxisporium*, obtained by somaclonal variation. Height reduction and large fruit size of selections 'LK-40' and 'LT-3', respectively, were found in Philippines by using gamma rays. In Sri Lanka, 'Embul-35 Gy' showed earliness by using gamma rays. IAEA also reported that 'GN35-I to GN35-VIII' were selected clones derived from the 'Grand Naine' tolerant to toxin of *M. fijiensis* by using gamma rays.

In banana, induced mutation technique by ion-beams has not been reported. In the present study, the ion-beams mutation induction technique was applied to study the effect of irradiation doses on banana explants and regenerated plantlets to black Sigatoka disease reaction. This

research is aiming at finding out the following objectives: 1) to identify the critical ion-beams doses; 2) to induce genetic variability for mutant selection and; 3) to evaluate the response of black Sigatoka disease on the irradiated plants.

4. 2. Materials and Methods

4. 2. 1. Assessment of banana explants in laboratory conditions

4. 2. 1. 1. Cultivars of banana

In this study, cultivars 'Williams' and 'Cavendish Enano' were irradiated on June 6, 2005. The second irradiation was conducted for 'Williams', 'Orito' and 'FHIA-01', on November 14, 2005

4. 2. 1. 2. Explants slicing conditioning for ion-beams irradiation

For irradiation, banana explants as thin as 2 mm were requested to allow entire penetration of the ion beam. Prior to irradiation, from April 28 to May 10, 2005, an experiment was conducted to clarify the regeneration rate of the thinner banana explants. Cultivars 'FHIA-01' and 'Williams' were used. Two types of slicing or cutting methods were applied to the banana corms shoot tips (both vertical and horizontal slices). The slices were placed in liquid medium for regeneration, containing MS medium, supplemented with BA (5 mg L^{-1}), and sucrose (20 g L^{-1}), at pH 5.6. Twenty-five slices were placed into a 300 mL Erlenmeyer flask containing 100 mL of medium. Five flasks (replications) containing 'Williams' and 'FHIA-01' explants were prepared. The explants were stirred in a shaker at 100 rpm. Regeneration rate and weight of explants

were recorded. From the results, the highest number of regenerated plants obtained using either of the two methods were selected and applied in the establishment of the experiment for irradiation.

4. 2. 1. 3. Ion-beams irradiation doses

Four-week old shoot tips from banana cultivars were used as a source of explants for *in vitro* propagation. The explants (from vertical slices) were placed in a 6 cm Ø plastic dishes containing the solid medium and were covered with sterilized Kapton films (8 μm thickness, Toray-Dupont, Japan) in order to prevent the loss of energy of the carbon ions. The samples were irradiated with doses of 0, 0.5, 1, 2, 4, 8, 16, 32, 64 and 128 Gy. The irradiation of each Petri dish was finished within 1 minute.

The explants were planted on the Petri dish 2 days before irradiation (June 4, 2005). For this purpose, 20 explants/dish x 2 dishes (40 explants per doses) were used, having a total of 400/per cultivar (800 explants considering the two cultivars). The procedure for explants preparation is shown in Fig. 22.

Two days after irradiation (June 8, 2005) the explants were transferred into a 100 mL Erlenmeyer flask containing 50 mL of the new MS liquid medium. Nineteen days later (June 27, 2005), the growth of the explants were evaluated using the following parameters: weight, height, survival rate and LD_{50}.

Second irradiation was conducted on November 14, 2005. Cultivars ‘Williams’, ‘Orito’ and ‘FHIA-01’ were used applying doses of 0, 0.5, 1, 2, 4, 8 and 16 Gy, considering that in the previous experiment the high dose affected drastically to the explants. In this case, shoot cluster’s weight, shoot cluster’s height, number of shoot cluster’s, survival rate and LD_{50} were recorded.

4. 2. 1. 4. Post-irradiation management

After the evaluation of the explants growth at the laboratory, each treatment/cultivar growing in the Erlenmeyer flask were propagated three times using the same composition of the liquid medium mentioned above, in order to increase the number of explants for nursery and field conditions experiments, that were conducted in Ecuador. They regenerated into shoot tips and then they were planted individually into a test tube containing 10 mL of MS solid medium. Additionally, 0.5 mg of activated carbon for rooting was added by applying on the solid medium surface. A total of 1707 rooted plantlets were sent to Ecuador (December, 2005) in autoclaved plastic bags. Processing plants for bagging is shown in Fig. 23.

The numbers of plantlets per cultivar/doses were as follows: for 'Williams' in the doses of 0 (94), 0.5 (95), 1 (105), 2 (95), 4 (32), 8 (87), 16 (103), 32 (70), 64 (95) and 128 Gy (60). For 'Cavendish Enano' in the doses of 0 (55), 0.5 (95), 1 (95), 2 (100), 4 (80), 8 (40), 16 (80), 32 (53), 64 (53) and 128 Gy (100). The materials were found free of viruses such as Banana Bunchy Top Virus (BBTV), Banana Bract Mosaic Virus (BBMV) and Banana Streak Virus (BSV) by using ELISA virus indexing technique. They also were free of any other pest and disease including *nematodes, Mycosphaerella fijiensis, Fusarium oxisporum*, and so forth.

The plantlets arrived in the Laboratory of Biotechnology of the Estación Experimental Tropical Pichilingue, Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), Ecuador. Immediately, the bags containing the plantlets were kept in the tissue culture room at 26 ^{o}C and light conditions for two days to recover photosynthesis. When arrived, the banana material showed injured probably occurred during transportation (Fig. 24).

4. 2. 2. Assessment of banana plantlets at the nursery and at field conditions

A soil bed was constructed inside a greenhouse and the plantlets were transferred on a soil substrate (soil : vermiculite = 1:1) and covered with a plastic sheet to avoid dehydration (Fig. 25). Factors such as transportation conditions, disease attack, or some abnormal behavior caused by the irradiation could bring about the high mortality (Fig. 26). Surviving plants were transplanted to bags and covered by using a cotton sheet and spraying water for three times a day to avoid plantlets dehydration (Fig. 27). Only 87 plants survived the nursery acclimatization. Plants were kept in a nursery plot until May 2006 and the bags were changed for a bigger one to recover vigor (Fig. 28). Fortunately, plants from every dose for the two cultivars survived except in the dose of 2 Gy of the ‘Cavendish Enano’. Using these materials (height of plant around 35 cm) the experiments for black Sigatoka inoculation in nursery plot and later on at the field conditions were conducted.

4. 2. 2. 1. Disease development period (DDP-days) and infection index (II-%)

Three banana leaves per plant were inoculated. Plants were about 30 cm height 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. Prior to inoculation of the first leaf, a solution containing a *M. fijiensis* conidial was prepared. The cultures were provided by the Laboratory of Pathology of the Estación Experimental Tropical Pichilingue, INIAP (Fig 29, A). The colonies for inoculation were cut in small pieces

and transferred to a 50 mL Erlenmeyer containing 20 mL of distilled water. A vortex was used to promote fast release of conidia into the solution. Once homogenized, the solution was filtered with 4 layers of gauze for collecting the filtrates in a flask (Fig 29, B). The solution was adjusted to 60 mL and then a concentration of 1.5 x 10^6/mL was obtained using a hemacytometer. Prior to inoculation, the sprayer was calibrated to determine the amount of solution to use. It was found that 45 mL of solution was enough to inoculate 87 plants, meaning that each plant was inoculated by about 0.5 mL of solution.

Before applying the inoculum to the plants, the old leaves were removed and the newly expanded leaf was selected and marked to be inoculated. Also the abaxial side of the marked leaf was cleaned using a soft cotton tissue. Using a small sprayer, the abaxial part of the leaf was sprayed with the solution in both sides (Fig 29, C). Immediately after the inoculation, the plants were kept in a dark incubation room for 48 hours adequate for the normal development of the fungus (Fig 29, D). The room provided continuous water aspersions to maintain a high relative humidity. The room temperature was 26^{o}C. After incubation, the plants were kept in a nursery plot for one week before transplanting to the field.

Due to the lack of monosporic cultures of *Mycosphaerella fijiensis* Morelet, the method to inoculate the second and third leaves was changed. The method consisted of applying completely diseased leaves fragments among the plantlets to produce high inoculum pressure. The leaves were collected from a banana collection located in the Estación Experimental Tropical Pichilingue, which have been kept without any chemical control for this disease (Fig. 30, A). Therefore, those leaves are considered as a potential natural inoculum capable to produce efficient sporulation pressure to inoculate the plantlets. Leaf-fragments were cut around 15 x 20 cm (Fig. 30, B) and they were sprayed with water prior to

placement among the plants (Fig. 30, C). Three leaf-fragments were placed per plant; two at the base of the plant and one inside the canopy for inoculation (Fig. 30, D). To ensure the inoculation of the plants, 170 leaf-fragments were placed on the flat at the nursery. All the leaf-fragments were sited exposing the abaxial side of the leaf to the plants.

As a preparation of the plants to be inoculated, the abaxial side of the leaf was cleaned by using a soft cotton tissue to remove the cuticular wax substance in order to permit a fast penetration of the fungus. The plants were grouped and the leaf-fragments inoculums were sited. Finally, by using a fickle cotton sheet the plants were covered to simulate an incubation chamber to ensure the sporulation and enhance the inoculation. The fickle cotton sheet was moistened thrice a day within 48 hours (Fig. 30, E). After this, the cotton sheet was removed. Around 10 days interval, both the second and the third leaf were inoculated using this method, however this inoculation also has an effect on the inoculation of the whole plant. After inoculation, the plants were kept in the nursery area and a week later the experiment was established at field conditions (June, 2006). 'Williams' vitroplants obtained from a commercial nursery were planted surrounding the experiment to avoid edge effects for the statistical analysis. The available numbers of plants per doses/cultivar were distributed in two replications (Fig. 31). To increase the pressure of inoculum in the environment where the plants were planted, diseased leaves from a banana collection belonging to the Estación Experimental Tropical Pichilingue were collected and placed near to the plants in the experiment (Fig. 32).

The disease development periods (DDP-days) was recorded, which consisted in the days between the inoculation time until the full development of the spot with dry gray center, using the stages of symptoms described by Fouré's scale and the disease severity determined by the infection index (II-%) calculated using the values obtained from the

Stover's scale modified by Gauhl (Orjeda, 1998) in which 0 = no symptoms; 1 = presence of spot (up to 10 spot, stage 4 of the Fouré scale); 2 = less than 5% of the leaf affected; 3 = from 6 to 15% of the leaf affected; 4 = from 16 to 33% of the leaf affected; 5 = from 34 to 50% of the leaf affected; and 6 = more than 50% of the leaf affected.

4. 2. 2. 2. Juglone toxin screening

Juglone (5-hydroxy-1,4-naphthoquinone) is one of the most active toxin among the seven toxins produced by *Mycosphaerella fijinesis* Morelet, that induce the formation of necrotic lesions of plants leaf cells (Strobel *et al.,* 1993). This toxin was used in this experiment to screen young plants as an indicator of resistance/tolerance to black Sigatoka disease. Previous results using different doses of juglone (Reyes-Borja *et al*., 2005) found that 150 ppm of this toxin was a suitable dose to get differences among the resistant cultivars and irradiated plants. The leaf-discs (5 discs per plant) were taken from the second expanded leaf of both ion-beams irradiated cultivars 'Williams' and 'Cavendish Enano' from the field planted experiment. Leaf-discs samples of 'FHIA-01' (resistant cultivar to black Sigatoka) were obtained from sucker plants available in the banana collection of the Estación Experimental Tropical Pichilingue, Ecuador.

One liter of solution containing 150 ppm of juglone was prepared using distilled water. An amount of 10 mL were dispensed into a Petri dish (90 mm) and the leaf-discs were immersed onto the solution. Leaves were rinsed with distilled water and 1.5 mm-diameter discs were removed using a cork borer (No. 8). Petri dishes with leaf-discs samples were kept on light and room temperature during 24 hours (Fig. 33). A total of 440 leaf discs were analyzed (87 plants x 5 leaf discs/plant). After 24

hours, photographs of the leaf-discs were taken by using a digital camera Minolta Dimage for necrotic areas percentage estimation. Photos were processed using the GIMP 2.2 software by selecting hand-drawn regions and their pixels. The percentage of necrotic area was calculated by the following formula:

$$\text{Disc necrotic area (\%)} = \frac{\text{Pixels of the full disc - Pixels of the green area}}{\text{Pixels of the full disc}} \times 100$$

4. 3. Results and Discussion

4. 3. 1. Assessment of banana explants in laboratory conditions

4. 3. 1. 1. Explants slicing conditioning for ion-beams irradiation

The analysis of variance for regeneration rate (%) reported high significance for slicing methods. The Tukey analysis ($P \leqq 0.05$), also reported significant differences between the slicing methods, but there are not significant differences among the cultivars. However, when the weight of the explants was analyzed, this analysis reported significant difference between cultivars.

The Fig. 34 shows the relationships of the explants weight (A) and regeneration rate (B) with the two slicing methods (vertical and horizontal) in ‘Williams’ and ‘FHIA-01’, 13-days after culture. Vertically sliced cuttings showed the highest regeneration rate (%) in both cultivars as is shown in Fig. 35. Values of 60-70% of regeneration rate were obtained in ‘Williams’ and ‘FHIA-01’ using the vertical cutting in contrast to only 37-43% in horizontally sliced cuttings. The weight of the explants were

similar in both cultivars when the corm shoot tips were cut vertically. In contrast, ‘Williams’ was affected when the horizontal slicing was applied. ‘Williams’ showed an average of 0.6 g using the horizontal slicing method, compared to 0.9 g using vertical slicing. Finally, the vertical slicing method was selected as a better method to regenerate the banana explants, and then this type of slice was obtained from shoot tips as a material for ion beam irradiation.

4. 3. 1. 2. Biological effects of the carbon ion-beam irradiation doses (Gy)

Results of the two cultivars (‘Cavendish Enano’ and ‘Williams’) used in the first irradiation are described as follow. The analysis of variance for the height of explant showed significant differences between cultivars and among ion-beams doses ($P \leqq 0.05$). As shown in Fig. 36-A and -B, the weight and height of plantlets were affected when the Gy doses were increased irrespective of cultivars. Highest growth behavior in terms of weight and height were observed when doses $\leqq$2 Gy were applied. These parameters decreased when 4 Gy or higher were used.

The survival rate (%) of the explants is presented in Fig. 36-C. In this study, it is supposed that the lower doses $\leqq$8 Gy had never interfered those survival rates even though the greater does resulted the higher mortality. Hase *et al.* (2002) reported that the high-linear energy transfer (LET) radiation such as heavy-ion beams have higher biological effects than low-LET radiation such as gamma and X-ray, producing a survival reduction and frequency of aberrant cells increased linearly. Thus, it is possible that the chromosome aberration induction depended on the LET. As shown in Fig. 37, the LD_{50} for both ‘Cavendish Enano’ (A) and

‘Williams’ (B) was obtained by exponential regressions. The LD_{50} for ‘Williams’ was at 13.5 Gy and ‘Cavendish Enano’ at 15.0 Gy, indicating the former would be more sensitive to the ion-beams than the latter. Figs. 38 and 39 shows banana plantlets of ‘Cavendish Enano’ and ‘Williams’ affected by different doses of ion beam (Gy), 19 days after irradiation.

The results of the three cultivars (‘Williams’, ‘Orito’ and ‘FHIA-01’) used for the second irradiation is mentioned as following. The analysis of variance for survival rate shows significant differences among the ion-beam doses, but there is no difference between cultivars. Tukey analysis ($P \leqq 0.05$) reported that there are two groups in which the means are not significantly different from one another. The analysis of variance for the numbers of shoot per explants reported significant differences between cultivars and among ion-beams doses, as well as Tukey analysis ($P \leqq 0.05$). The analysis of variance for the height of shoot reported significant differences between cultivars but there is not any difference among ion-beams doses. In contrast, the analysis of variance for the weight of shoot reported significant differences among ion-beams doses, but there is not difference between cultivars.

Fig. 40 is shown the shoot cluster’s weight (A), shoot cluster’s height (B), number of shoot cluster’s (C) and survival rate (D) of ‘Williams’, ‘Orito’ and ‘FHIA-01’ exposed to different doses of carbon ion-beams. The survival rate of the explants were similar when 0-8 Gy were applied, but 16 Gy this variable was affected. The numbers of shoot per clusters were different in each cultivar. ‘Orito’ showed higher values up to 8 Gy, however ‘Williams’ showed higher values when 16 Gy were applied. Shoot cluster’s weight and shoot cluster’s height showed a similar tendency across the ion-beams doses. Regenerated explants and cluster are shown in Fig 41.

Fig. 42 shows the LD_{50} for ‘Williams’, ‘Orito’ and ‘FHIA-01’ obtained by exponential regressions. The results shows that the LD_{50} for these 3 cultivars, were found out at 9.0, 3.8 and 4.6 Gy, respectively, indicating that ‘Orito’ and ‘FHIA-01’ were more sensitive to the ion-beams than the ‘Williams’. However, ‘Williams’ have been irradiated twice, showing 13.5 Gy and 9 Gy, respectively in the first and second irradiation. Even that, the first and second irradiation has reported high resistance to ion-beams irradiation compared with ‘Orito’ and ‘FHIA-01’.

In consequence, considering the data from the first and second irradiation, plus/minus (±) 5% was aggregated to each LD_{50} values of each cultivar to designate the optimum irradiation doses of carbon ion-beam Then, the optimum doses for ‘Williams’ ranged among 12.8-14.2 Gy, ‘Cavendish Enano’ (average of the two irradiation) ranged among 14.3-15.8 Gy, 4.4-4.8 Gy for ‘FHIA-0’1 and 3.6-4.0 Gy for ‘Orito’. Fig. 43 shows the optimum range for carbon ion-beams irradiation in each cultivar. LD_{50} of the cultivars are indicated in bold squares.

4. 3. 2. Assessment of banana plantlets at the nursery and at field conditions

4. 3. 2. 1. Disease development period (DDP-days)

The results of this variable were obtained from the regenerated plants from the first irradiation. DDP-days of ‘Cavendish Enano’ showed significantly longer rather than those of ‘Williams’. Among the three inoculated leaves in both cultivars, DDP-days were found shorter in the latest evaluations being significantly different ($P \leqq 0.05$). Figs. 44-A and 45-A shows the frequency distribution by class limits of the DDP-days in ‘Cavendish Enano’ and ‘Williams’ respectively. In these figures the higher

class limits values are clearly separated from the lower as is indicated between dotted vertical lines. In ‘Cavendish Enano’ highest DDP-days values ranged from 52.0 to 59.9 days and in ‘Williams’ varies from 50.0 to 54.9 showing quite different reaction expression against to black Sigatoka comparing with the lowest values in both cultivars.

All these values gave a sign that there exist great inter-individual variations of the DDP-days due probably to variable mutagenesis caused by the ion-beam irradiation. Molina and Castaño (2003) found in their results on inoculation methods using young plants with both resistant and susceptible cultivars to black Sigatoka, which the DDP-days in the resistant cultivar ‘FHIA-01’ was 77.5 days and the susceptible cultivar ‘Gros Michel’ was 31.2 days. In the present study, the high range of DDP-days demonstrated that there was a reaction of this group of plants against to the fast development of the disease. Additionally, Mobambo *et al.* (1997) reported differences of the DDP-days (identical with DDT by Mobambo *et al.*) between young and mature plants in a hybrid progeny of plantain. DDT of the hybrids were 7-16 and 28-36 days longer in young and mature plants, respectively. This variable allowed segregation between susceptible and resistant clones and also between different levels of resistance among the hybrids. These authors also mentioned that the similarity in ranking and response levels as shown by the high correlations between clones of mature and young plants indicate reliable and efficient evaluation in germplasm at young plants phase on response to black Sigatoka. However, agronomic traits like yield performance cannot be predicted by early evaluation for disease response of a clone.

In the present experiment, the variation of the DDP-days in each inoculated leaf could be due probably to the high inoculum pressure existing in the open field or the inoculum produced by the diseased leaves

in the plant itself. After inoculation of the leaf No. 1, plants still remained on the nursery until the inoculation of the subsequent leaves (No. 2 and 3). Nursery conditions were different as compared with the open field resulting in the variability of DDP-days. In contradiction, Mobambo *et al.* (1997) with a hybrid progeny obtained by crossing resistant and susceptible plantain cultivars reported that the disease assessment of subsequent leaves on young plants could reflect host response to black Sigatoka dependent on plant age. Symptom development on earlier produced leaves was faster than on later emerged leaves. Also another factor could be due to the inoculation method in the present study. As we mentioned above, the second and third leaves were inoculated using diseased leaf-fragments that could produce much inoculum capable to promote a fast DDP-days. However; Leiva *et al.* (2002) reported that mycelial homogenate and fragments of diseased leaves as types of inoculum of *M. fijiensis*, promoted disease symptom development in both 'Grande Naine' and 'FHIA-18' in greenhouse condition. Additionally, the susceptibility of 'Grande Naine' with respect to the partial resistances shown by 'FHIA-18' in greenhouse condition was found to coincide with the response of both cultivars in natural conditions.

4. 3. 2. 2. Infection index (II-%)

The results in this variable were from the regenerated plants subjected to the first irradiation. II-% showed significant differences between the cultivars and the evaluation periods (June 16, 27, July 10 and 24, 2006) by Tukey analysis ($P \leqq 0.05$). 'Williams' possess highest II-% than 'Cavendish Enano'. The II-% was found higher in latest evaluations. Figs. 44-B and 45-B are showing the II-% frequency distribution by class limits of "Cavendish Enano' and 'Williams', respectively. Lowest II-%

values in ‘Cavendish Enano’ ranged between 25.0 to 34.9 days and 27.0 to 36.9 days in ‘Williams’ as is marked between dotted vertical lines showing pretty variation contrasting greatly with the higher values.

Cohan *et al.* (2003) working with a plantain hybrid ‘CRBP-39’ (AAAB) which resulted from across by a female triploid ‘French clair’ (AAB) susceptible to black Sigatoka and the banana diploid ‘M53’ (AA) resistant cultivar, obtained very low infection index in three phases (at vegetative 6-month, flowering time and harvest), having values of 0.50, 0.08 and 0.0% of this parameter, respectively, as a character of excellent resistant to black Sigatoka as compared to the parental female which showed 33.37, 22.47 and 91.46%, respectively. Molina and Castaño (2003) with ‘FHIA-01’ and ‘FHIA-17’ lowest values of II-% were obtained during the vegetative growth, flowering and harvest (3 phases) in comparison with the susceptible as it is ‘Gros Michel’. The two former ones showed 33, 32 and 41% at three phases; in contrast the latter that were 46, 44 and 90%. At the harvest phase functional leaves were 5 in both resistant cultivars compared with no leaf obtained in the susceptible cultivar. These results substantiate the positive relationships between the functional leaves and bunch weight. In terms of resistance mechanism, Romero and Sutton (1997) mentioned that the exact mechanism of resistance of bananas to black Sigatoka is unknown. Low stomatal density and increased levels of epicuticular wax, phytoalexin production, the production of lignin or suberin, and resistance to phytotoxins produced by pathogen have been suggested as possible mechanisms. Krishnamoorthy *et al.* (2004a) reported that the levels of chlorophyll, sugars, and the activity of ascorbic acid oxidase were positively correlated with the infection index, whereas the levels of proline and lignin, and the activity of peroxidase were negatively correlated.

4. 3. 2. 3. Juglone toxin response

The analysis of variance reported not significant differences between cultivars. Fig. 44-C and Fig. 45-C are showing the LDNA-% frequency distribution by class limits of “Cavendish Enano’ and ‘Williams’, respectively. Lowest II-% values in ‘Cavendish Enano’ varied from 38.0 to 44.9 days and 33.0 to 39.9 days in ‘Williams’ simply observed between dotted vertical lines. Additionally, LDNA-% values from non-irradiated plants did not show values as lower as 44.0 % in ‘Cavendish Enano’ and 38% in ‘Williams’. Fig. 46 shows high (A) and low-affected (B) leaf discs by juglone. Buiatti and Ingram (1991) mentioned that leaf discs technique have been mainly used for early screening of segregation population within classical breeding program, or to direct *in vitro* selection of tolerant cells. Leaf-disc bioassays have been reported 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, however it should only serve as a preliminary screening technique before field test. Sackston and Vimard (1988) used the leaf-discs immersion technique to study a host-pathogen interaction to distinguish resistant or susceptible sunflower lines to *Plasmopara halstedii*.

Other techniques such as micro-cross sections of banana plantlets also have been used to select plants *in vitro* culture levels. Since Okole and Shulz (1996) obtained high regenerable callus and plantlets from micro-cross section of banana and plantain, they applied this method to select *M. fijiensis*-resistance cell lines. Okole and Shulz (1997) studied a double selection system; at first, micro-cross section was performed to access the growth into a crude fungal filtrate and then the regenerated shoot bud was subjected to the purified fungal toxin 2,4,8-trihydroxytetralone.

Only a partial resistance was observed in most of the regenerated plantlets after toxin selection. In addition, Stierle *et al.* (1991) isolated six different phytotoxin compounds from *M. fijiensis*, having different biological activity for each. The 2,4,8-trihydroxytetralone (tetralone) exhibited host selectivity comparable to that of the fungal pathogen at the 5µg/5µl level, following by juglone that was stronger than tetralone in terms of toxicity.

In this research, it seems that the ion beam irradiation promoted mutation in cells, reacting different when they were confronted with the toxins. On the other hand, Etame (2003) characterizing *M. fijiensis* toxins by using a high performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS) revealed the presence of juglone, but did not reveal any of the *M. fijiensis* metabolites already reported. Once isolated this toxin, banana genotypes possessing different reactions were used to compare their susceptibility to *M. fijiensis* metabolites and their sensitivity to the infection by the pathogen. The most resistant genotypes to juglone ('Fougamou', 'Pisang madu', 'M53' and 'Klutuk') appeared also resistant to the infection, although some cultivars resistant to *M. fijiensis* are susceptible to juglone. Globally these results suggest that juglone could allow to identify the more resistant genotypes to the infection of *M. fijiensis*. Lepoivre *et al.* (2002) described that 250 ppm of juglone is required to induce necrosis in the leaf puncture bioassay using selected plants from embryo cell suspension of 'Three hand planty', however, the selected plants did not show higher resistance to black Sigatoka than the mother plant.

Tables 10 and 11 shows the raw data obtained in the variables DDP-days, II-% and LDNA-% for 'Cavendish Enano' and 'Williams', respectively.

4. 3. 2. 4. Plant selection by combining DDP-days, II-% and LDNA-%

In this research, the three variables DDP-days, II-% and LDNA-% were study to assess black Sigatoka response in the irradiated materials. The data analyzed by linear regression permitted to categorize the plants showing better response against to this disease. Fig. 47 shows the LDNA-% regression versus II-% (A), LDNA-% regression versus DDP-days (B) and DDP-days regression versus II-% (C) in 'Williams'. The regression among the three combined variables permitted to observe close relationships in six plants. The code number are 'W 16 II 74', 'W 128 I 67', 'W 1 II 148', 'W 8 II 13', 'W 1 II 19' and 'W 1 II 31'. This group of plants were selected as candidates possessing better response against to black Sigatoka. In the case of 'Cavendish Enano', two plants with the code numbers CE 4 II 30 and CE 64 I 5 (Fig. 48) were found out showing high relationship when the three variables were combined (Table 12). Those results allow to allege that the sensitivity to the irradiation of 'Williams' determined by LD_{50} could explain the variation in the number of mutated plants that shows six candidates, compared with the two candidates obtained in 'Cavendish Enano'. That means that high sensitivity cultivars to Carbon ion-beams irradiation may produce a wide range of mutagenesis expressing tolerant/resistant to black Sigatoka, deducing that the sensitivity is cultivar dependent.

Romero and Sutton (1997) indicated that artificial inoculations of 10-week-old tissue culture plants with conidia of *M. fijiensis* could be used to test the susceptibility or resistance of different genotypes. In this study, which is the first report in banana using ion beams to alter the banana response against to black Sigatoka, from a viewpoint of complete assessment of the candidate plants, field experiment based on the whole plant cycle must be necessary to evaluate not only the response to black

Sigatoka but also fruit quality, potential production and postharvest parameters as valuables components for final selections.

4. 3. 2. 5. Relative DNA content

When 115 samples were analyzed by using flow cytometer, hexaploid cells were found out. In 'Cavendish Enano' five plants were found when the ion beam dose was 4 Gy. In Cultivar 'Williams' just one plant was observed (Fig. 49). These results suggested that carbon ion-beam irradiation could produce a duplication of the chromosomes. Yu (2006) in the experiment with wheat mentioned that if a normal chromosome increased by a segment with the same sequence could be called duplication. The duplicated segment is linked to the original sequence, it is called tandem duplication; if the segment is linked in the reverse sequence, it is called a reverse duplication. The same author also reported that a large amount of chromosomal lagging can definitely induce change in the chromosome number and thus possible result in new idioplasms of aneuploid. Using N-ion implantation doses of D3 (3×10^{16} ions/cm^2) and D4 (4×10^{16} ions/cm^2), monomers with the chromosomes number 41 appeared in wheat 'Premebi'. In 'Rye AR1' with dose D4, monomers with the numbers of chromosomes of 19 appeared, 15 of them being normal chromosomes and 4 as B chromosomes which were obviously a trisome.

4. 3. 3. Factor of effectiveness produced by carbon ion-beams

Factor of effectiveness (FE) of 'Cavendish Enano' and 'Williams' are showed in Tables 13 and 14. Plants showing good response to black Sigatoka were selected. Other useful characteristics such hexaploids plants and fast growth plantlet were also observed.

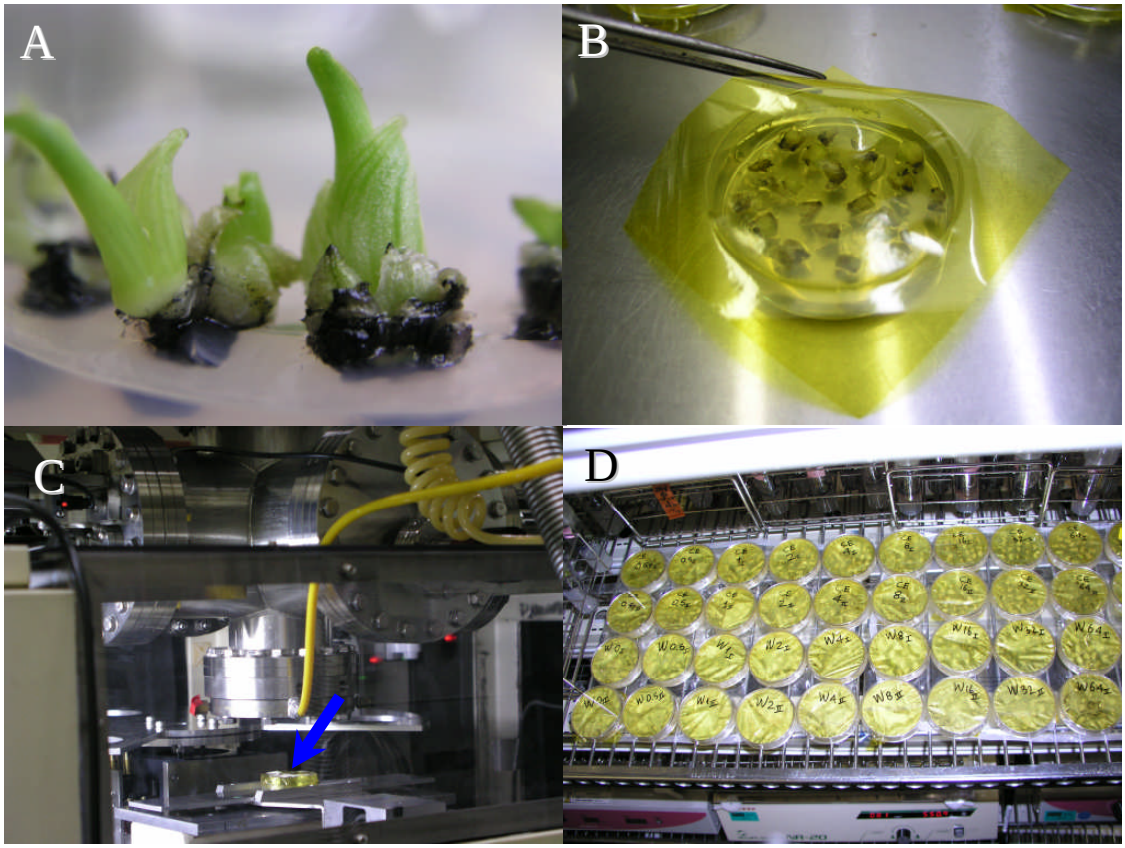

Fig. 22. Preparation of the banana explants before irradiation. Shoot tips as explants source (A). 20 explants (sliced)/dish covered by Kapton films, 8 μm thickness autoclave (B). Dish placed (indicated by the arrow) on the carbon ion-beams irradiator (C). 40 dishes were irradiated, 800 explants in total (D).

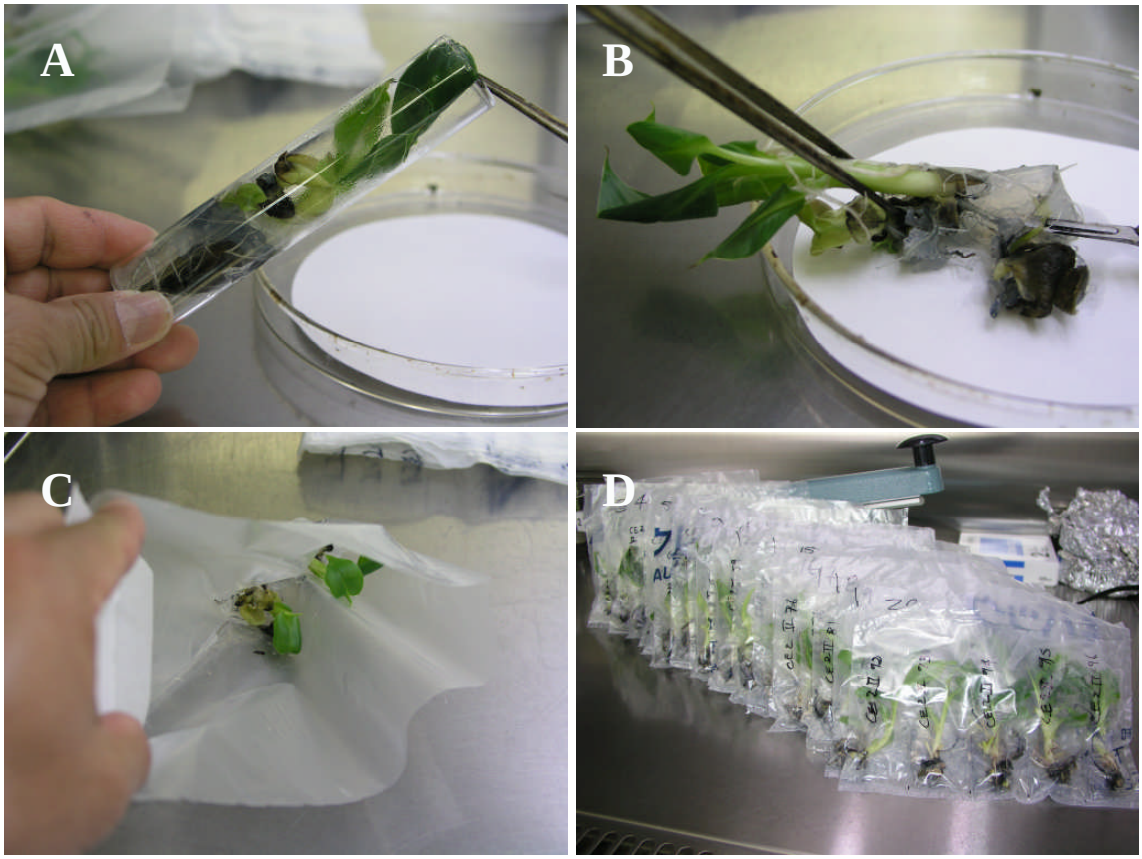

Fig. 23. Banana plantlets packaging for transportation. Plantlets growths into a test tube (A), removing medium and black root and leaf (B), introducing the plantlets into the sterilized bags (C), and packaged plantlets, 5 per bag (D).

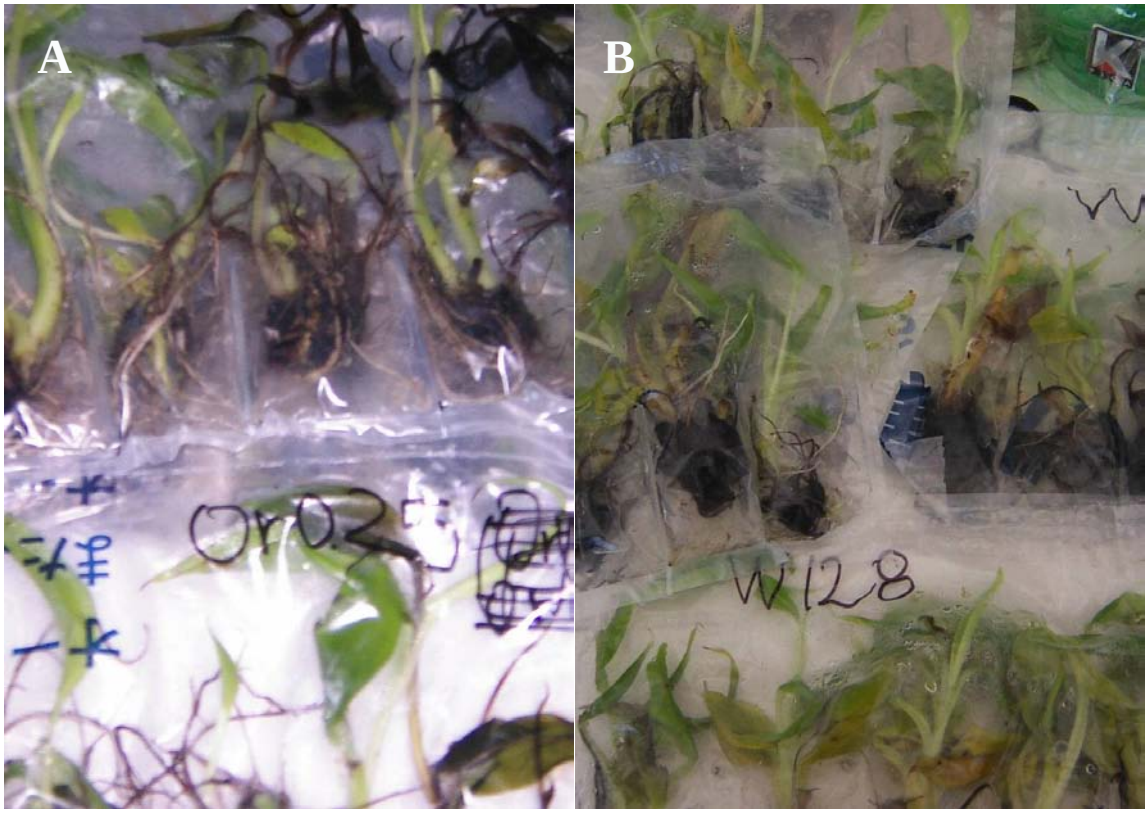

Fig. 24. Plantlets injured during transportation to Ecuador. Cultivars ‘Orito’ (A) and ‘Williams’ (B).

Fig. 25. Acclimatization of the plantlets at greenhouse condition. Mixing soil and vermiculite (A), opening bags for planting (B), plantlets on the bed soil (C) and plantlets with plastics cover to avoid dehydration (D)

Fig. 26. Plantlets for acclimatization at the green house condition. Plantlets after planting (A), Plantlets 10 days after plating affected by fungal attack (B-D).

Fig. 27. Survival plantlets for acclimatization on the nursery plot. Remind plants after fungus attack (A), removing plantlets for planting (B), bags with a mixture of soil containing the plantlets (C) and covered plantlets using a cotton sheet to avoid dehydration (D).

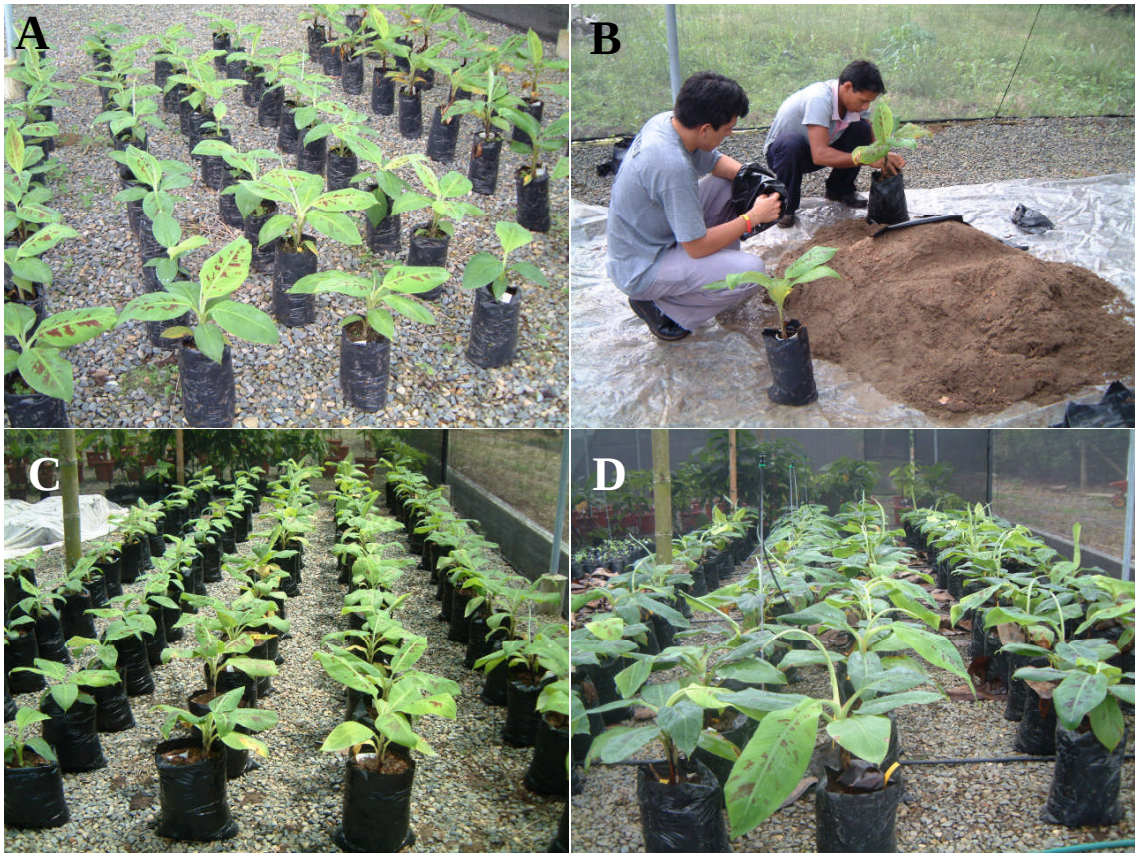

Fig. 28. Survival plants at the acclimatization process (A). Plant kept at the nursery plot (May, 2006). Changing the plants in a bigger bag using soil mixture (B). Plants in bigger bags (C), and recovered plants one month later (D, June, 2006).

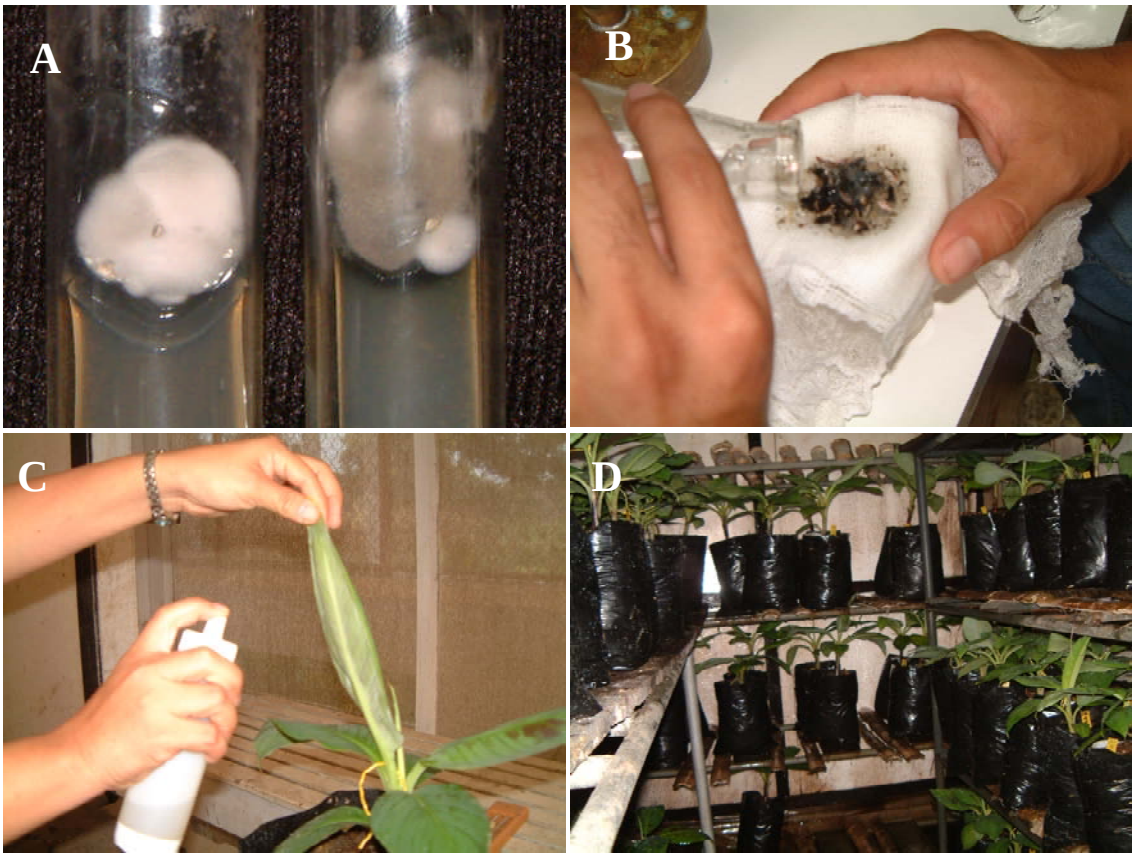

Fig. 29. *Mycosphaerella fijiensis* Morelet colonies (A), filtration (B), and inoculation of the irradiated banana plants (C) kept in an incubation room during 48 hours (D).

Fig. 30. Inoculation method using leaf diseased fragment. Collected leaves from a banana collection kept in the Estacion Experimental Tropical Pichilingue, INIAP, Ecuador. Leaves disease fragments moistening before placement among the plants to be inoculated (B, C, and D). Covered plants y a cotton sheet for 48 hours, and moistening thrice a day (E). Plant showing the inoculated leaves (F).

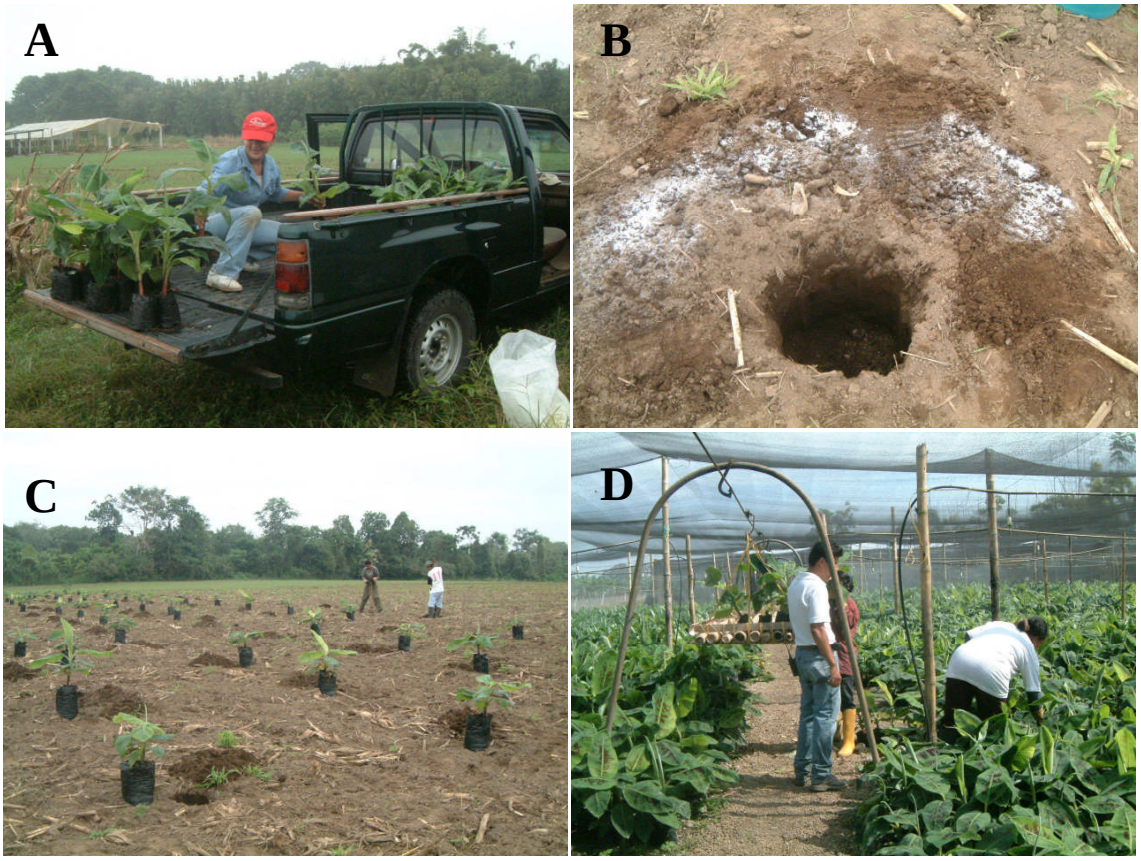

Fig. 31. Labor during transplanting irradiated banana plants (A-C). ‘Williams’ plantlets were obtained from a commercial nursery to planting surrounding the experiment to avoid edge effects (D).

Fig. 32. After planting, diseased leaves were placed besides de plants to increase the inoculums pressure (A-D).

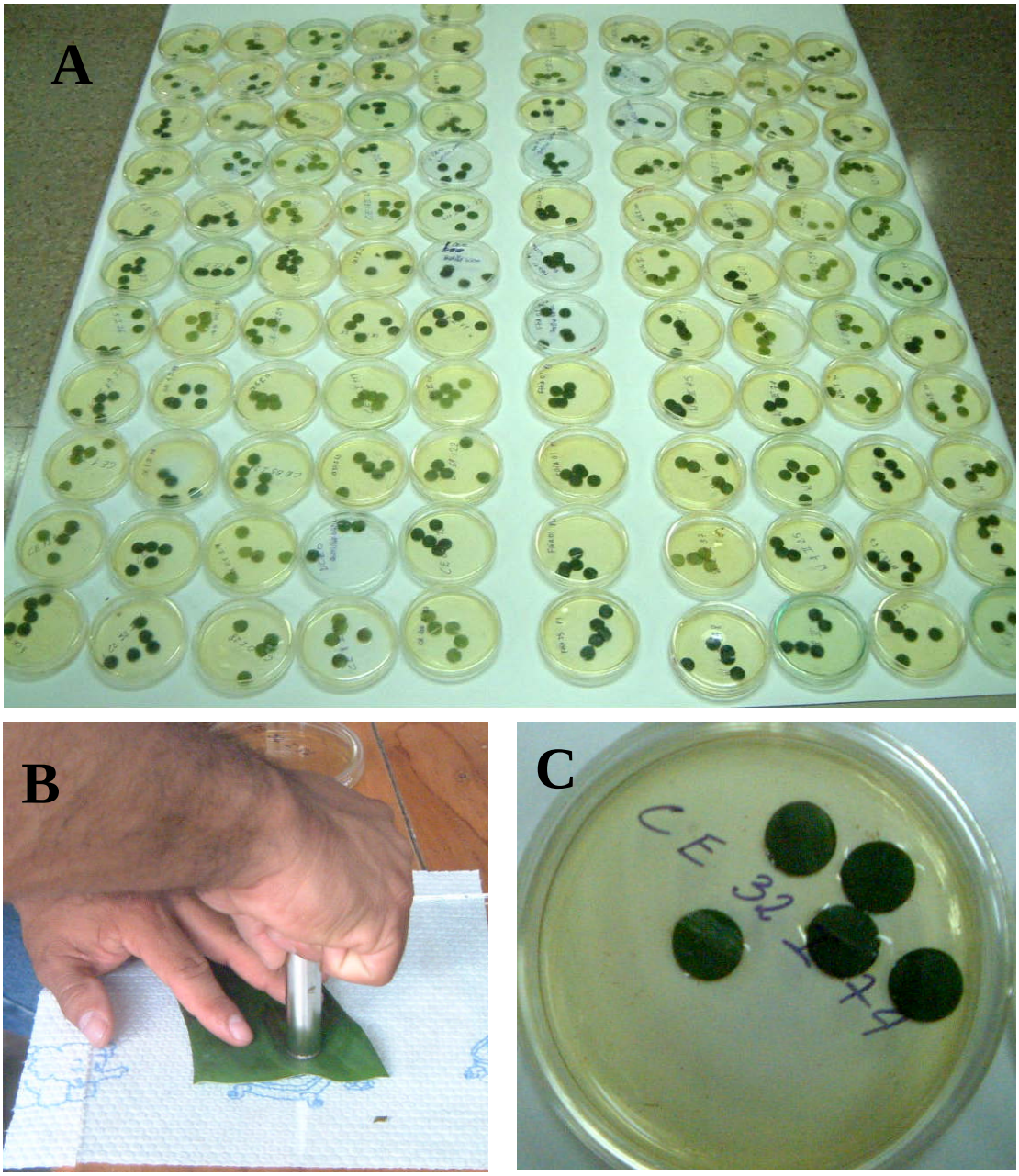

Fig. 33. Establishment of the juglone toxin experiment using leaf discs method (A-C).

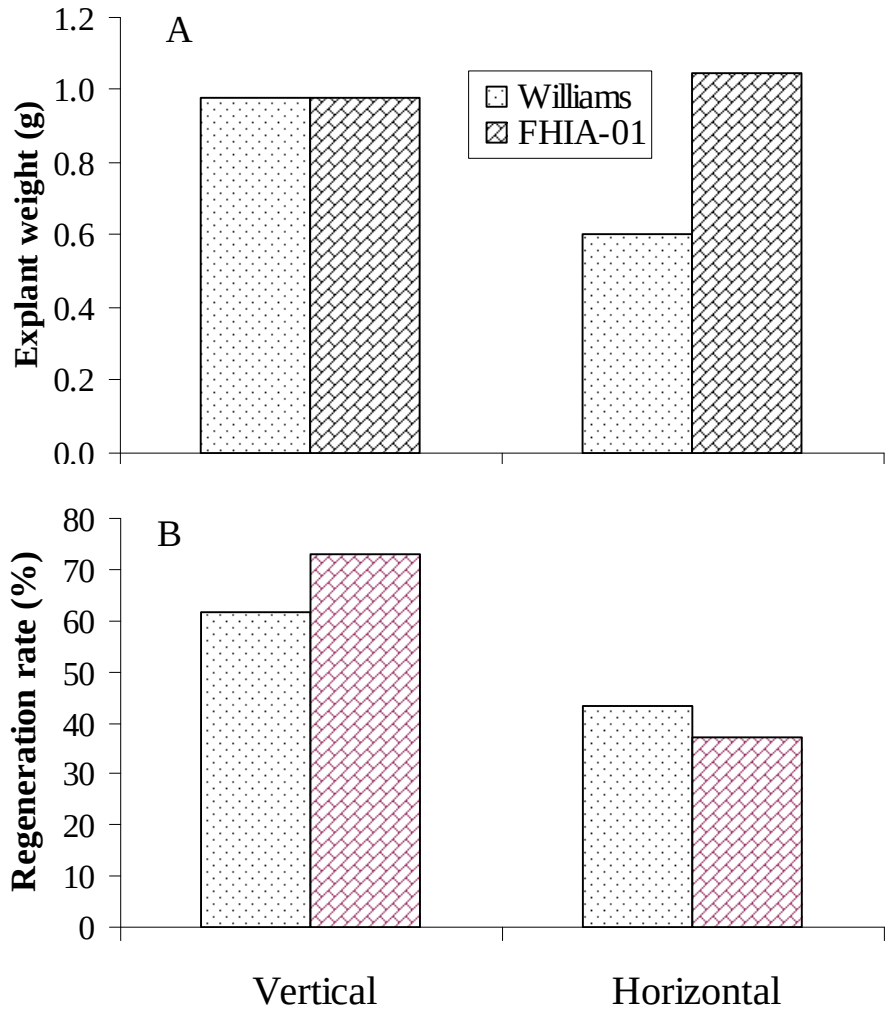

Fig. 34. Relationships of the explants weight (A) and regeneration rate (B) with the two slicing methods (vertical and horizontal) in 'Williams' and 'FHIA-01', 13-days after culture.

Fig. 35. Regenerated plantlets of ‘Williams’ from both vertical (A) and horizontal (B) slicing methods 13 days after culture.

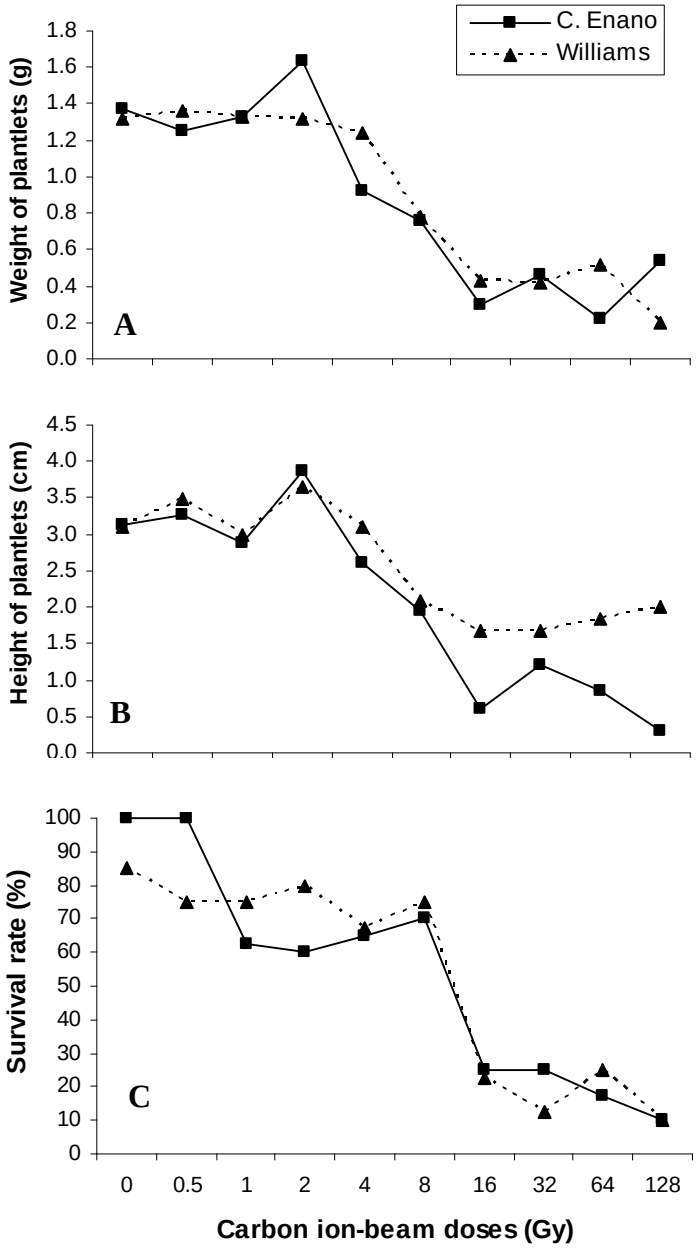

Fig. 36. Weight (A), height (B) and survival rate (C) of the banana plantlets in both 'Williams' and 'Cavendish Enano' cultivars, 19 days after ion beam irradiation.

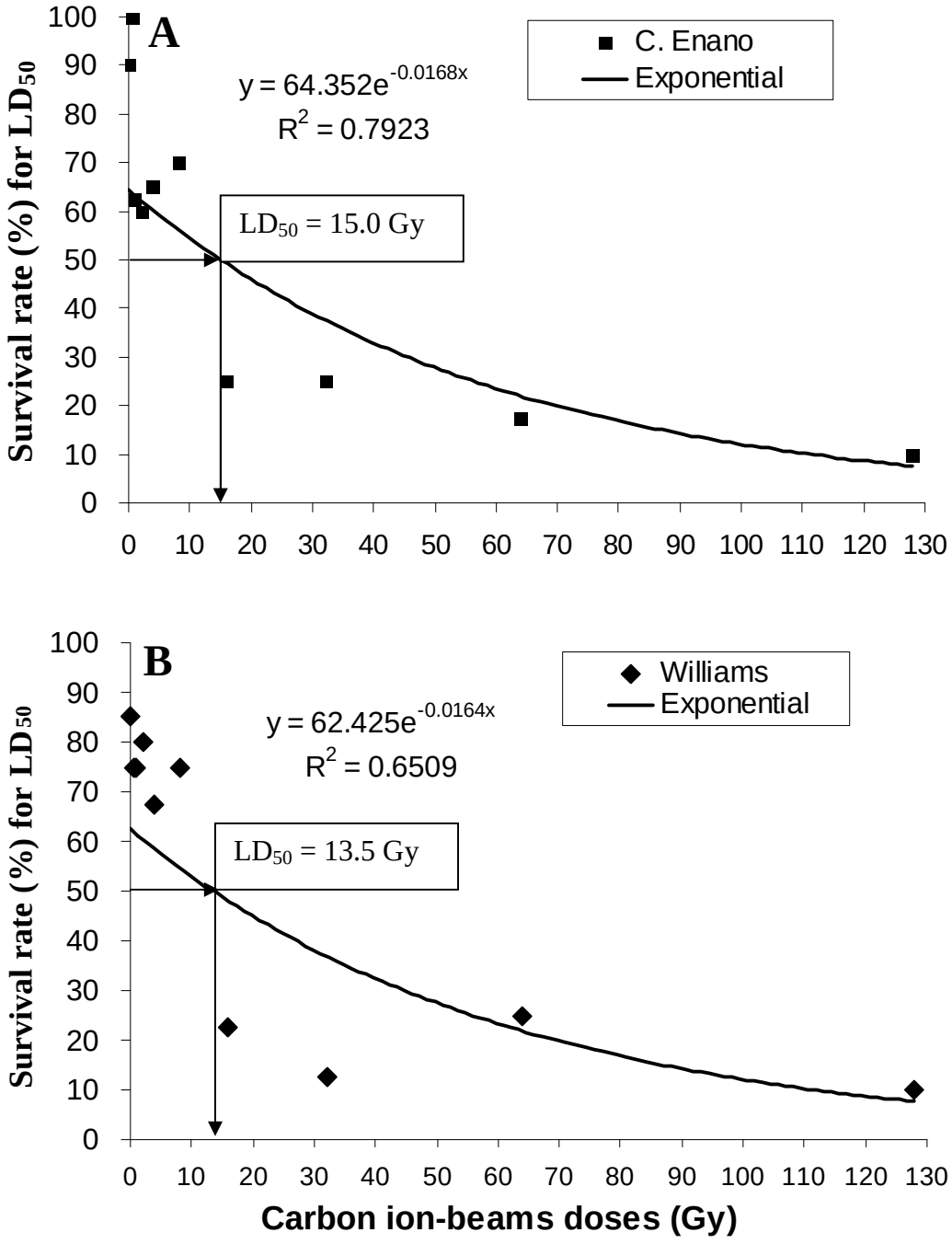

Fig. 37. Exponential regression of dose response and survival rate for LD_{50}, in both 'Cavendish Enano' (A) and 'Williams' (B). Arrows shows the LD_{50} for both cultivars.

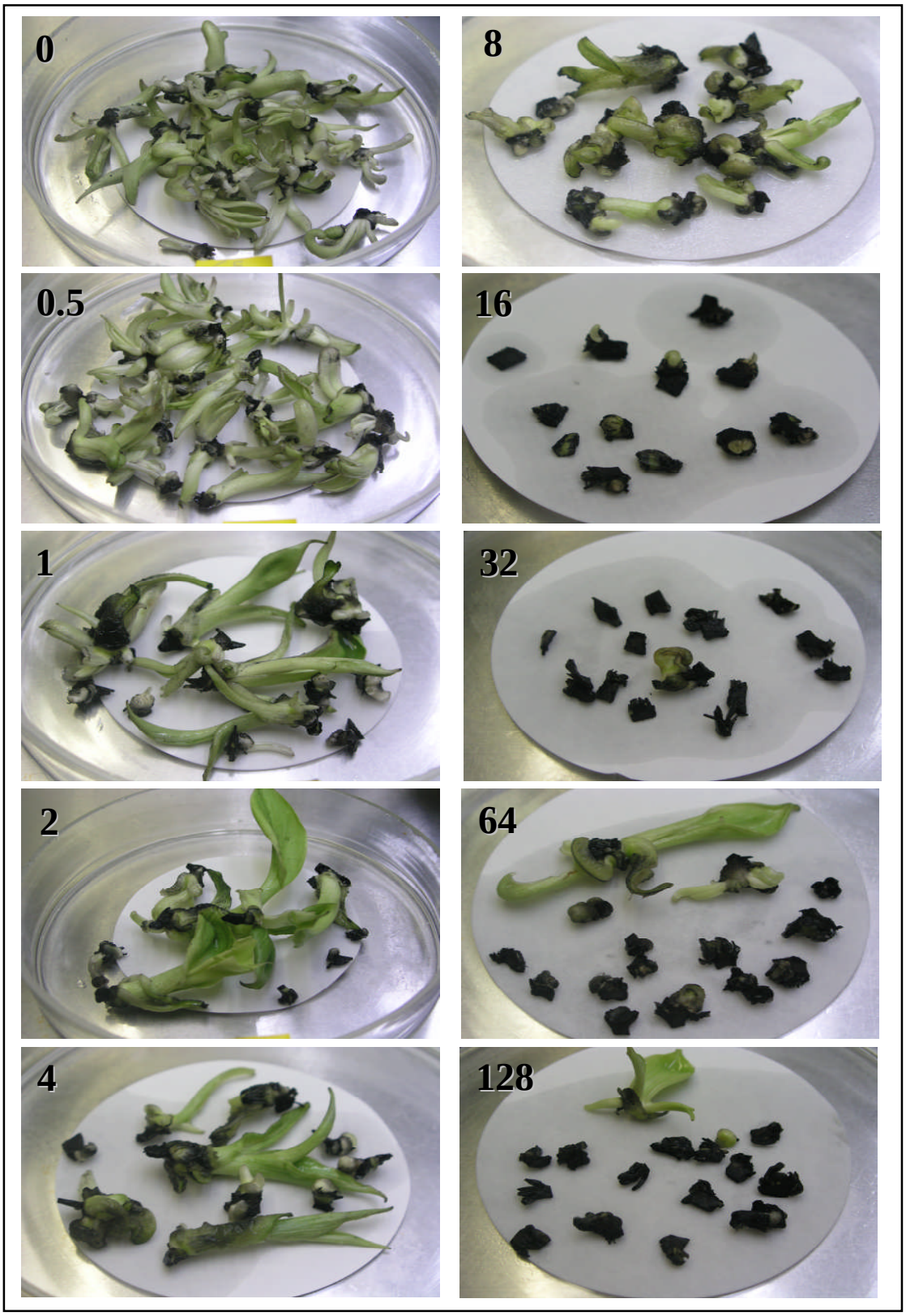

Fig. 38. Banana plantlets of ‘Cavendish Enano’ affected by different doses of ion beam, 19 days after irradiation.

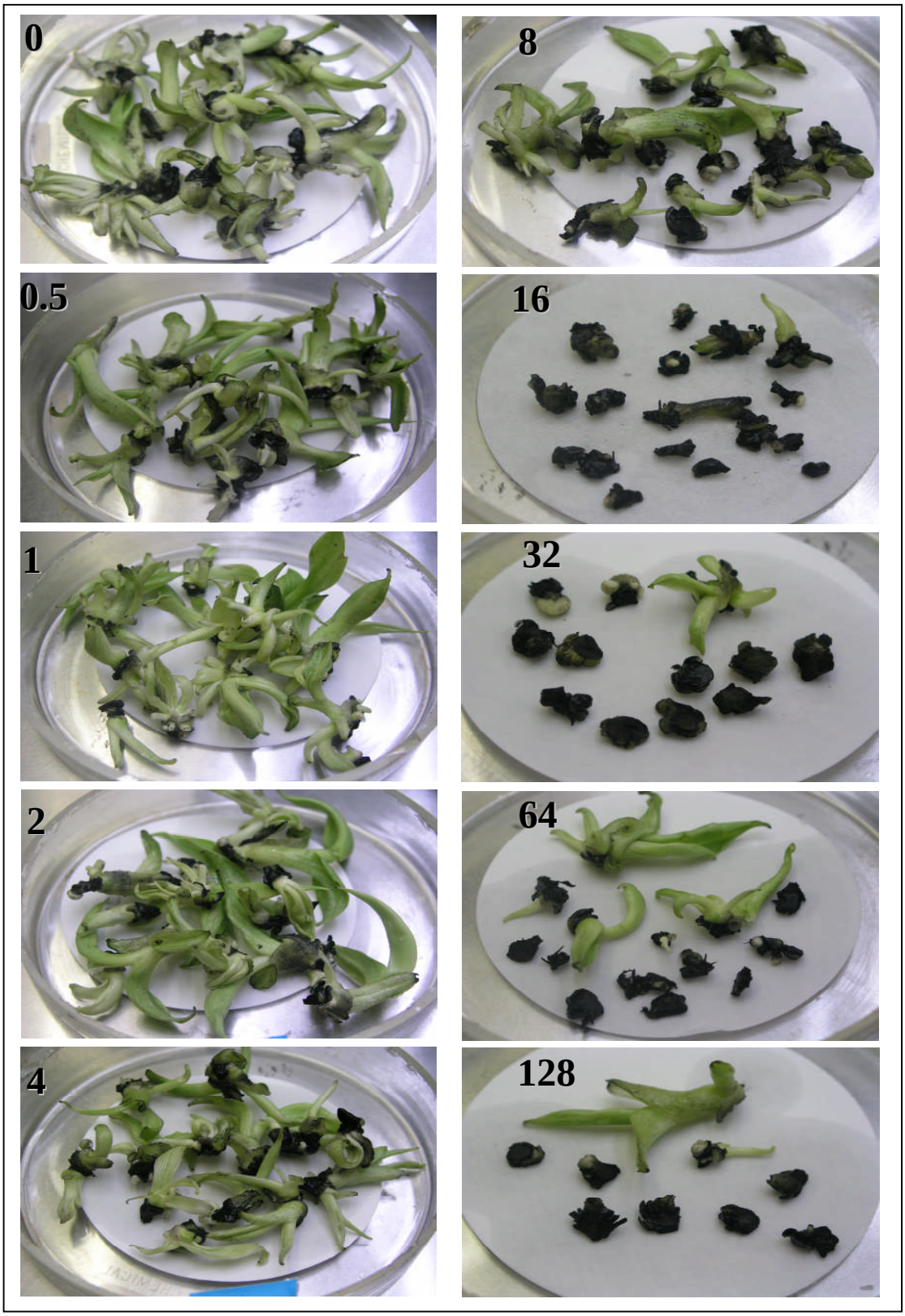

Fig 39. Banana plantlets of ‘Williams’ affected by different doses of ion beam (Gy), 19 days after irradiation.

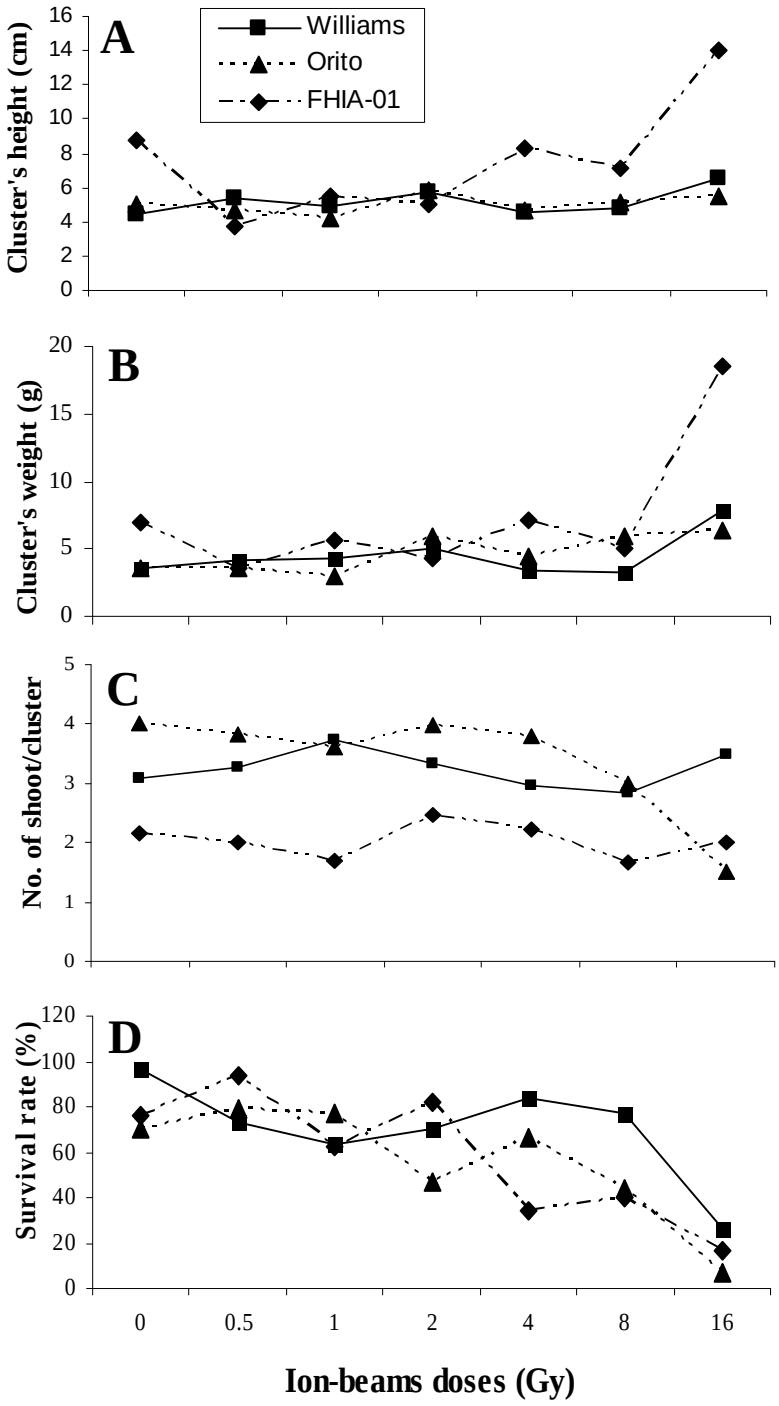

Fig. 40. Shoot cluster's weight (A), shoot cluster's height (B), number of shoot cluster's (C) and survival rate (D) of the banana plantlets in 'Williams', 'Orito' and 'FHIA-01'.

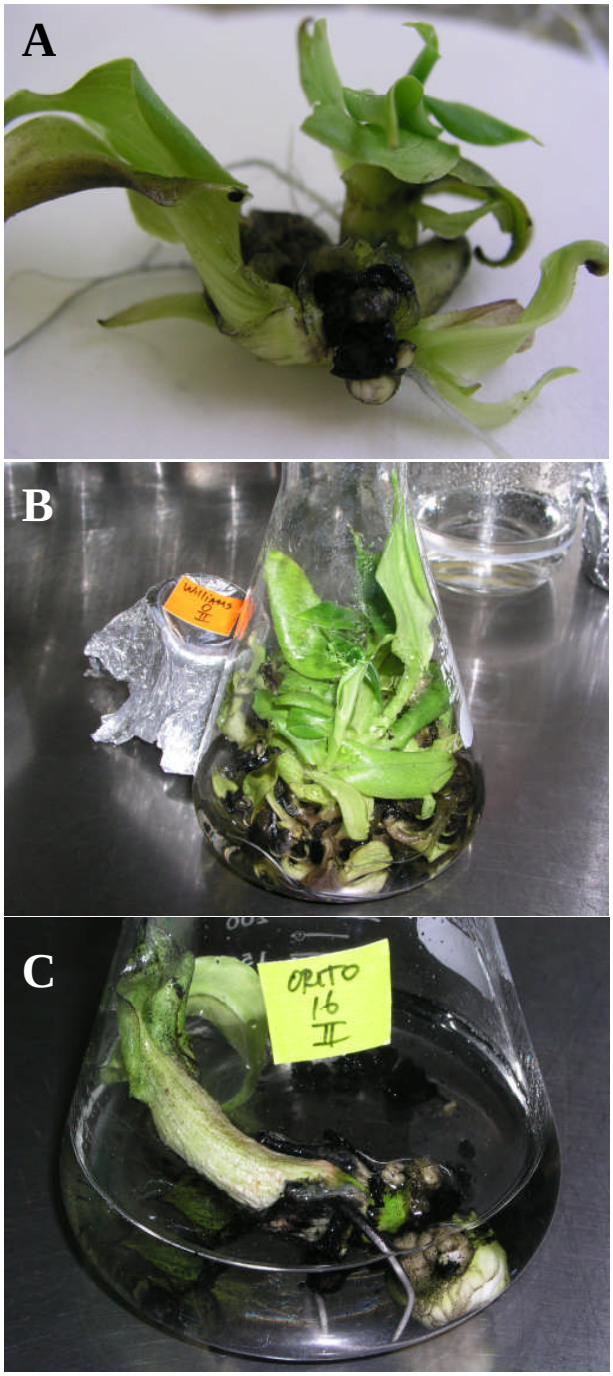

Fig. 41. Regenerated explants from second ion-beams irradiation. Shoot cluster (A). Normal growth of the clusters (B) and low survival when 16 Gy were applied (C).

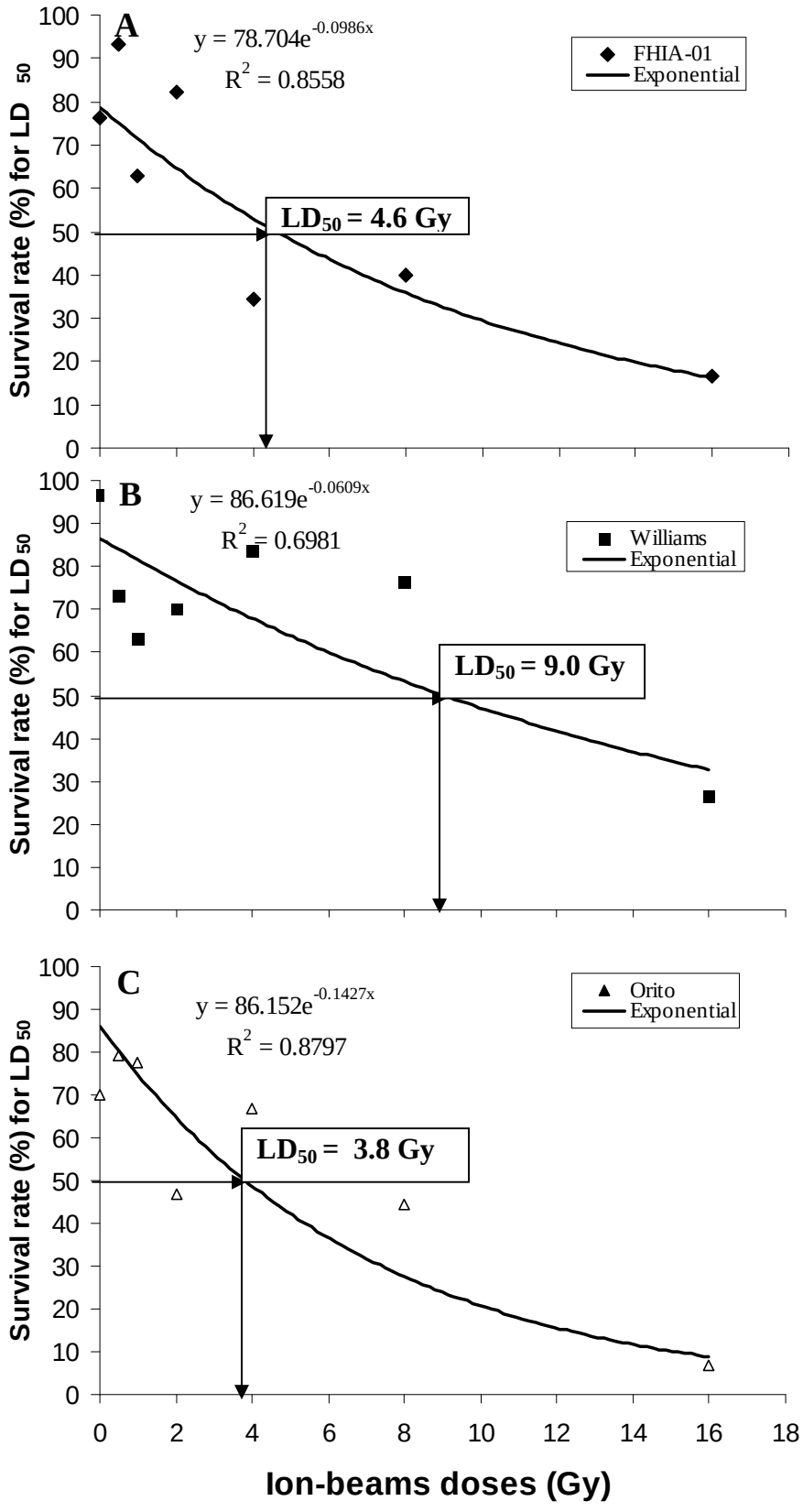

Fig. 42. Exponential regression of dose response (Gy) and survival rate (%) for LD_{50}, in 'FHIA-01' (A), 'Williams' (B) and 'Orito' (C). Arrows show the LD_{50} for the three cultivars.

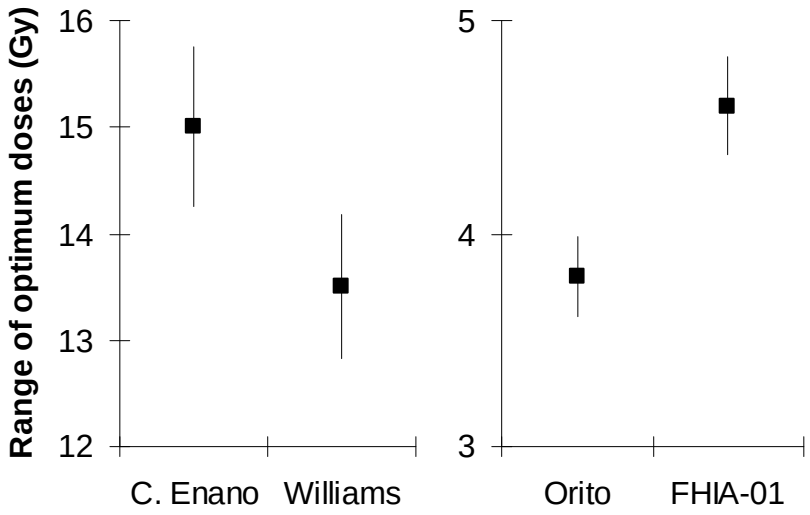

Fig. 43. Range of optimum carbon ion-beams doses (Gy) obtained by plus/minus (±) 5% of the LD_{50} values. Bold squares represent the LD_{50} values.

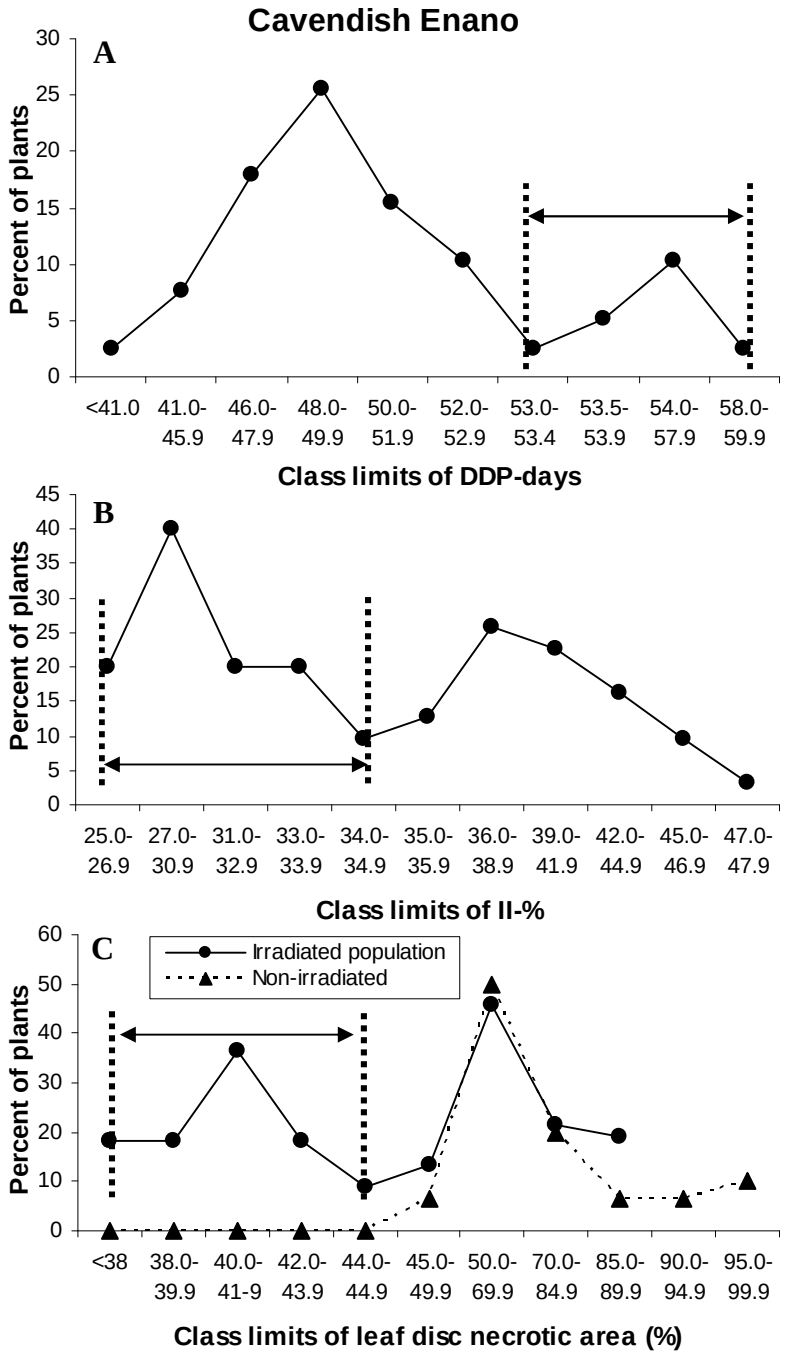

Fig. 44. Efficiency of mutagenesis occurred after ion-beam irradiation as expressed by frequency distribution by class limits of DDP-days (A), II-% (B) and LDNA-% (C) in 'Cavendish Enano'. A range between dotted vertical lines indicates the best values of the three parameters.

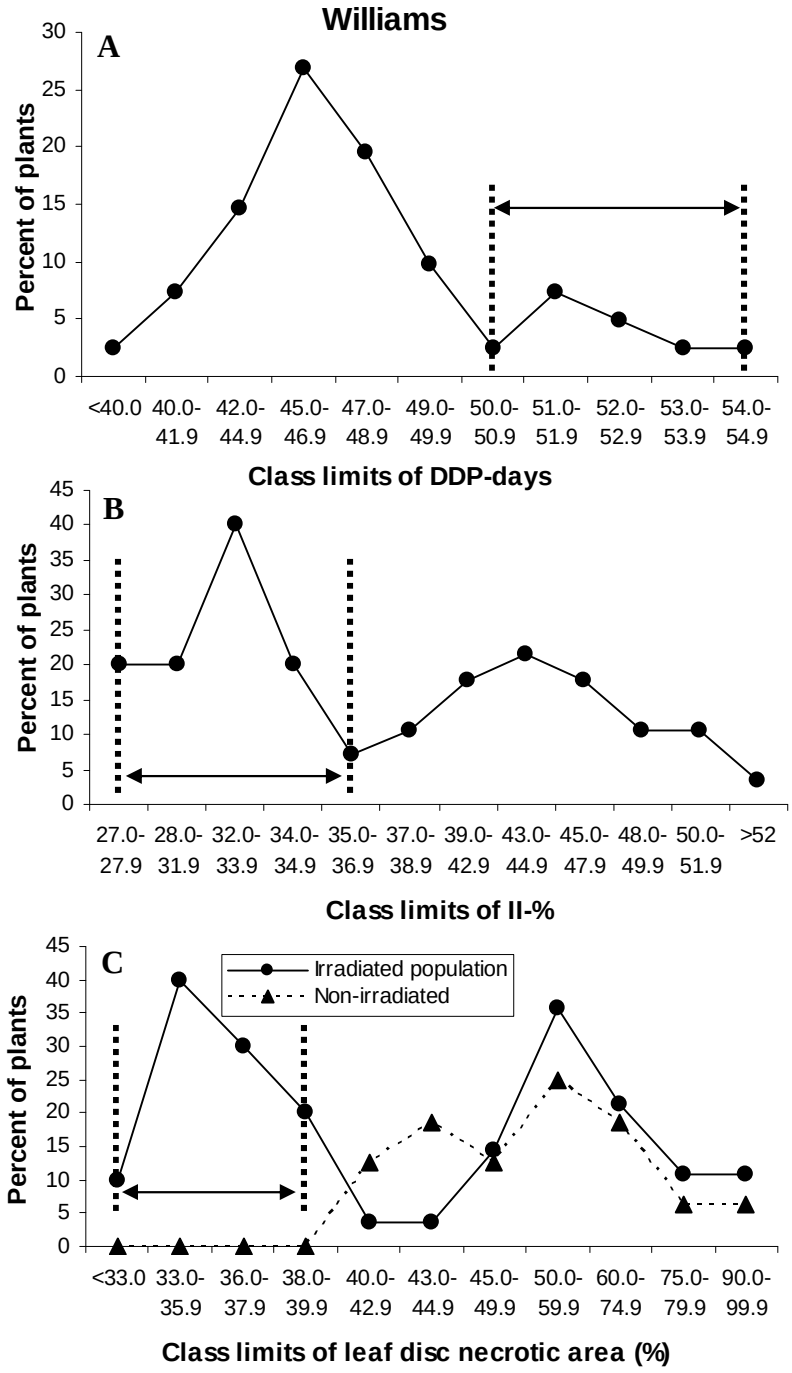

Fig. 45. Efficiency of mutagenesis occurred after ion-beam irradiation as expressed by frequency distribution by class limits of DDP-days (A), II-% (B) and LDNA-% (C) in ‘Williams’. A range between dotted vertical lines indicates the best values of the three parameters.

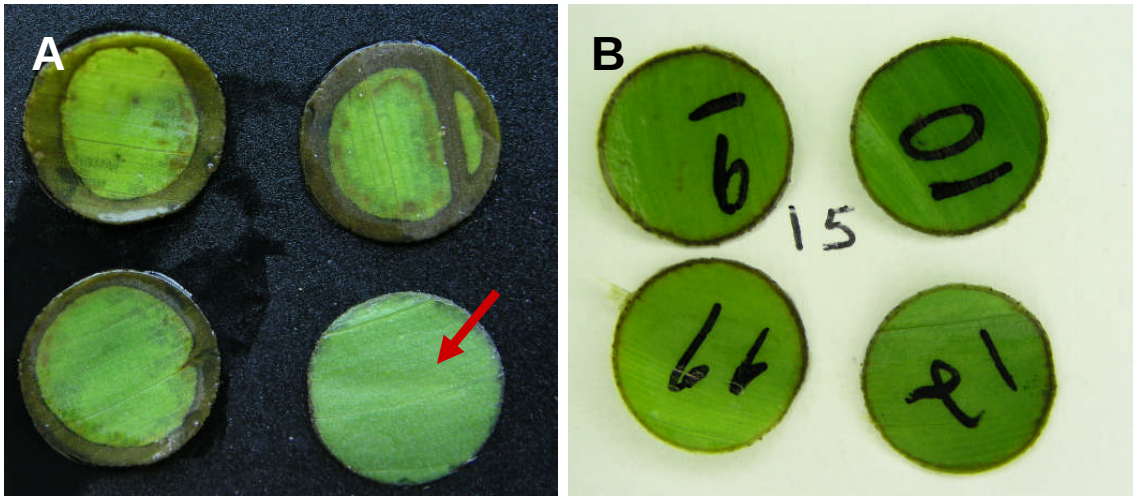

Fig. 46. Leaf-discs screening method to select juglone tolerant plants. High percentage of leaf-discs necrotic area affected by juglone (A). Juglone low-affected leaf-discs (B), Arrow shows a leaf-disc treated with distilled water.

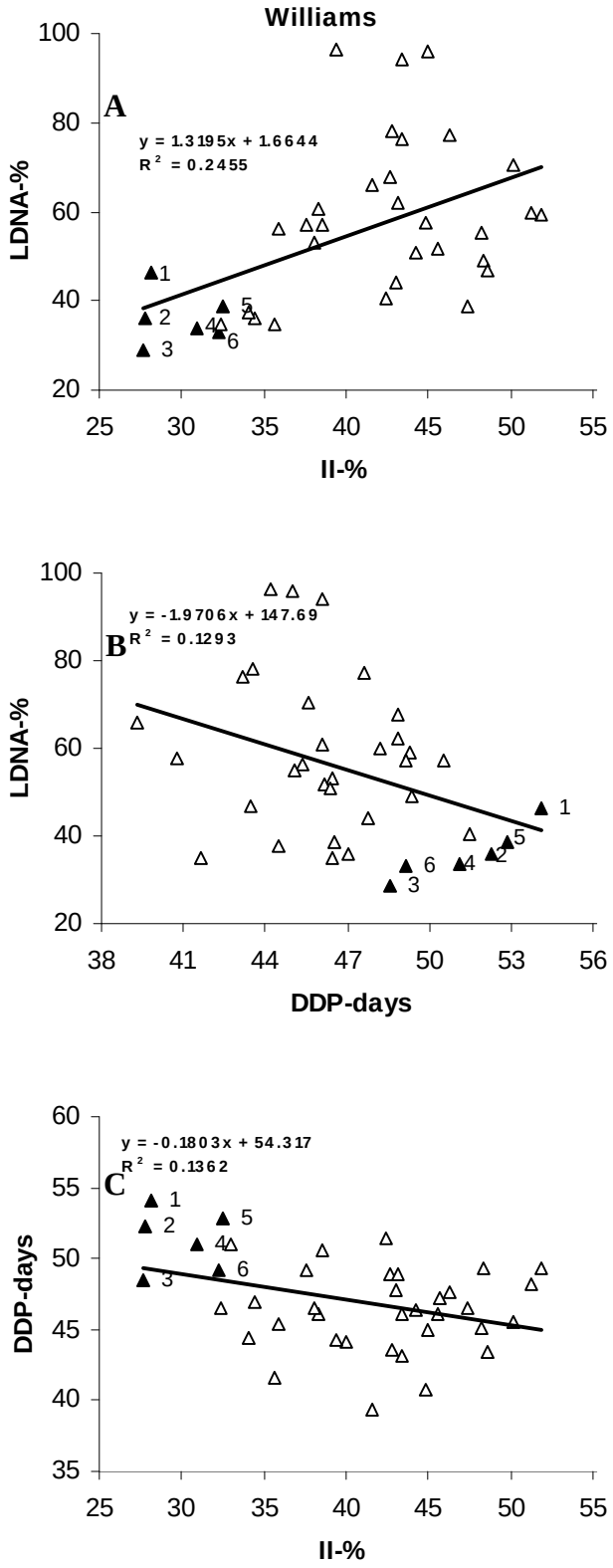

Fig. 47. LDNA-% regression versus II-% (A), LDNA-% regression versus DDP-days (B) and DDP-days regression versus II-% (C) in 'Williams'. Values in bold triangles with the code number 'W 16 II 74' (1), 'W 128 I 67'(2), 'W 1 II 148'(3), 'W 8 II 13'(4), 'W 1 II 19'(5) and 'W 1 II 31'(6) clearly shows relationships within the three combinations of the variables.

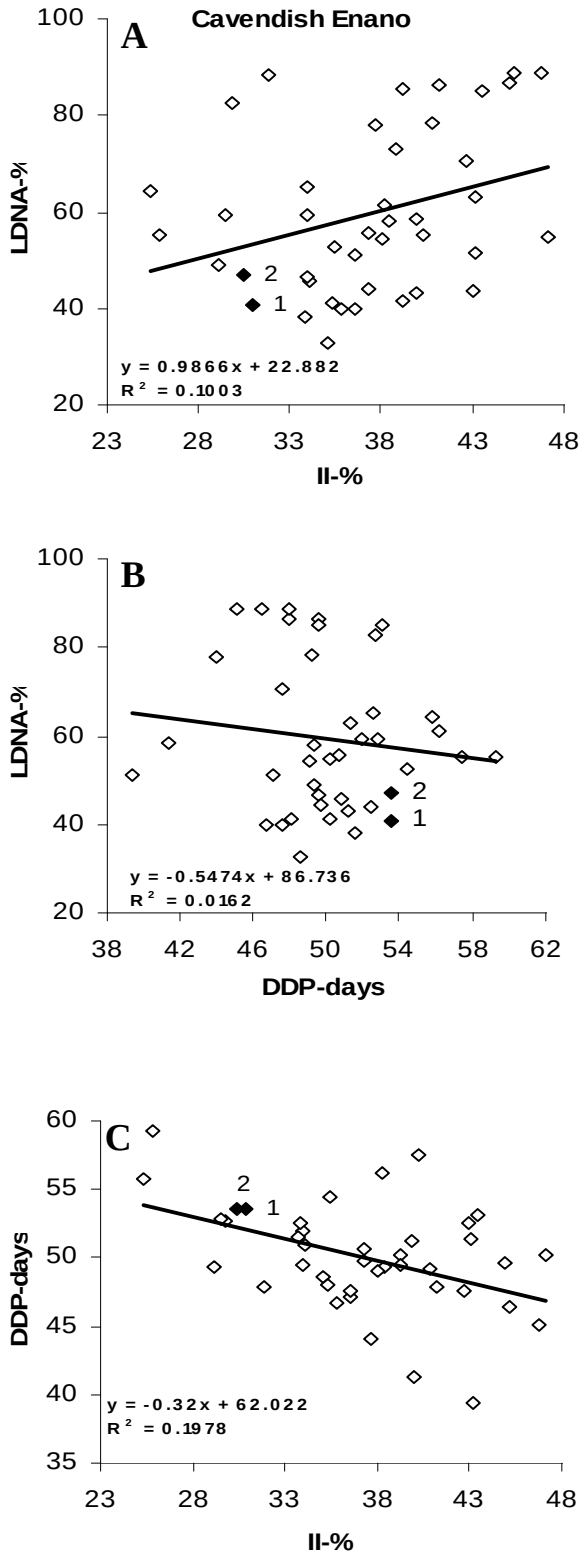

Fig. 48. LDNA-% regression versus II-% (A), LDNA-% regression versus DDP-days (B) and DDP-days regression versus II-% (C) in 'Cavendish Enano'. Values in bold diamond with the code number 'CE 4 II 30' (1) and 'CE 64 I 5' (2) clearly shows relationships within the three combinations of the variables.

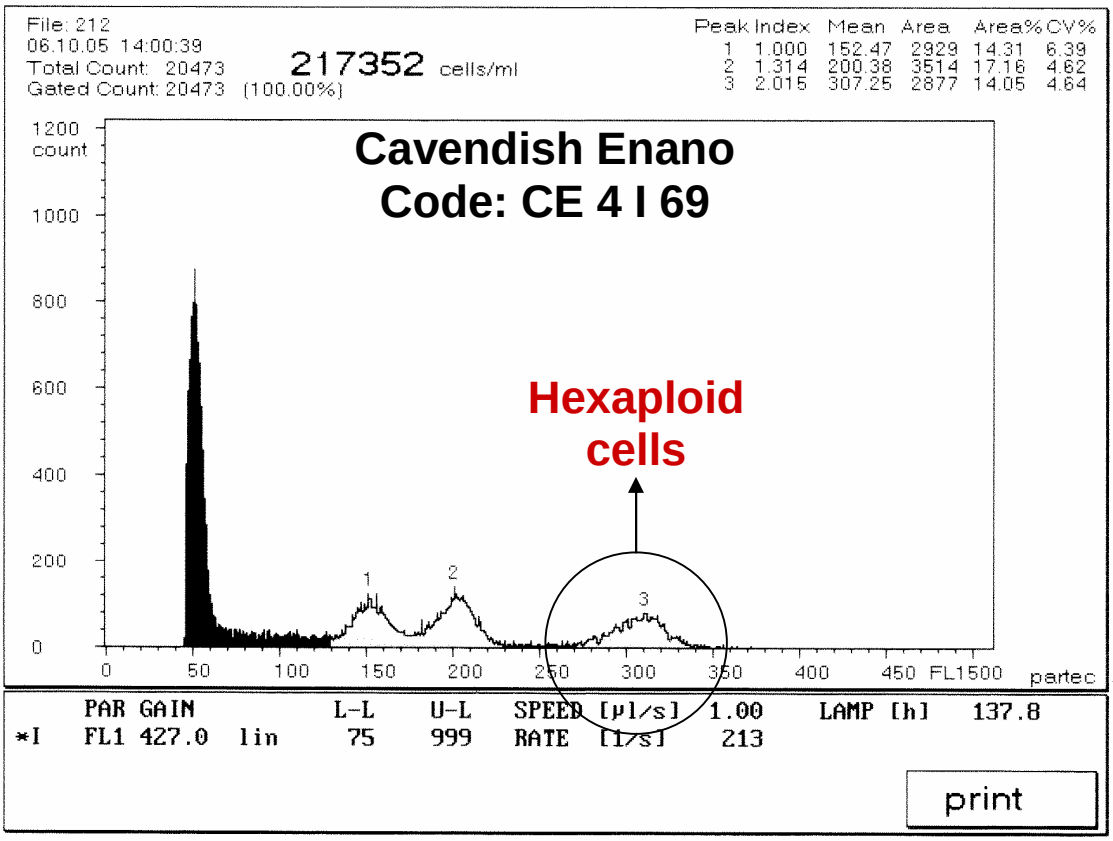

Fig. 49. Hexaploid cells in ‘Cavendish Enano’ (encircled) produced by ion beam dose 4 Gy.

Fig. 50. A ‘Cavendish Enano’ plant from carbon ion-beams irradiation (4 Gy) showing a fast growth (center) compared with the all plants in the group.

Table 10. Infection index (II-%), disease development period (DDP-days) per inoculated leaf and leaf-discs necrotic area (LDNA-%) in ion-beams irradiated 'Cavendish Enano' plantlets.

Cultivar/code No.	Black Sigatoka-infection index (%)/evaluation				DDP/inoculated leaf No.			
Cavendish Enano	16-Jun-06	27-Jun-06	10-Jul-06	24-Jul-06	1	2	3	
CE 0 I 28	18.5	33.3	37.5	64.3	57.6	52.6	38.0	58.09
CE 0.5 I 9	27.8	20.8	33.3	54.2	61.4	55.4	39	59.32
CE 0.5 I 1	20.4	31.5	41.7	87.5	53.2	51	35.2	88.84
CE 0.5 II 14	8.3	21.4	52.4	73.3	47.8	-	-	73.22
CE 0.5 II 59	24.1	33.3	27.8	56.7	67	55	41.2	52.72
CE 0.5 I 24	24.1	35.0	33.3	68.8	63	60.2	49.2	55.15
CE 1 I 26	31.5	25.0	43.8	59.5	61.4	51	41.2	43.18
CE 1 II 61	18.5	31.8	37.0	29.2	53.2	46.8	48	49.02
CE 1 II 98	20.4	25.0	47.9	47.2	51.2	48.2	46.4	33.07
CE 1 II 28	24.1	25.0	41.7	83.3	59	48.2	52.0	84.93
CE 1 I 14	27.8	18.3	53.7	72.2	54	51	-	43.75
CE 4 I 66	25.9	31.3	41.7	61.1	49	44	31.0	58.54
CE 4 II 30	20.8	25.9	33.3	43.8	60.6	54.2	46.0	40.69
CE 4 II 88	27.1	20.8	52.1	43.3	51.2	46.8	42.0	40
CE 4 II 126	19.0	38.9	38.9	66.7	60	49.6	38.0	78.4
CE 8 II 11	20.8	33.3	52.4	58.3	56.2	49.6	38.0	86.23
CE 16 II 17	11.1	20.8	42.6	52.8	54.8	51	38	88.57
CE 16 II 28	14.8	37.5	58.3	76.7	50.4	49.6	35.2	88.62
CE 16 II 10	24.1	35.0	55.6	58.3	51.2	40	27.0	51.33
CE 16 II 25	25.0	22.2	29.2	25.0	60.6	51	-	64.29
CE 32 I 69	31.5	31.3	47.6	40.5	50	44	44.4	78.05
CE 32 I 86	20.8	36.7	54.8	76.7	45.8	51	38.0	51.03
CE 32 I 86	11.1	30.0	45.8	59.5	60	51	39.6	54.83
CE 32 II 115	14.6	37.5	47.9	80.0	59.8	51	38.0	86.55
CE 32 I 74	22.9	14.6	42.9	76.7	57.6	51	42.0	41.44
CE 32 I 63	16.7	20.4	37.5	61.1	62.2	53.4	42.0	65.28
CE 64 II 39	22.2	37.0	41.7	70.0	55.4	45.4	42	70.43
CE 64 II 112	16.7	31.5	44.4	80.0	59	51	44	63.01
CE 64 I 122	22.2	20.4	35.8	70.8	59.8	54.2	38	55.73
CE 64 I 15	14.8	16.7	45.8	59.3	57.6	51	52	45.67
CE 64 I 117	20.8	12.5	37.5	48.3	59	51	48.0	82.73
CE 64 I 13	20.4	18.5	44.4	51.9	57.6	51	46.0	38.1
CE 64 I 5	14.8	28.3	35.7	42.9	65	49.6	38.0	46.68
CE 64 II 109	24.1	30.0	45.8	41.7	51.2	51	42.0	41.13
CE 128 I 84	11.1	35.2	35.7	75.0	57.8	49.6	41.2	85.29
CE 128 I 7	18.5	28.8	40.5	48.1	54.4	51	43.2	46.61
CE 128 I 95	9.5	18.8	31.3	43.9	63	63	52.0	55.14
CE 128 II 72	20.8	22.2	35.0	40.0	63	54.2	41.2	59.38
CE 128 I 119	24.1	33.3	55.6	33.3	57.6	45.4	39.6	39.69
CE 128 I 87	22.9	25.9	37.5	66.7	63	53.4	52.0	61.25
CE 128 II 74	33.3	22.9	35.4	57.4	56.2	51	42.0	44.21
CE 128 II 71	24.1	27.1	41.7	59.5	56.5	51	39.6	54.49

Table 11. Infection index (II-%), disease development period (DDP-days) per inoculated leaf and leaf-discs necrotic area (LDNA-%) in ion-beams irradiated 'Williams' plantlets.

Cultivar/code No.	Black Sigatoka-infection index (%)/evaluation				DDP/inoculated leaf No.			Leaf-discs necrotic
Williams	16-Jun-06	27-Jun-06	10-Jul-06	24-Jul-06	1	2	3	area (%)
W 0 II 45	-	25.0	50.0	40.5	54	49.6	48.0	57.20
W 0 II 39	27.1	42.6	50.0	40.5	46.8	51	34.6	-
W0.5 II 30	22.2	21.4	42.9	66.7	44	48.2	46.0	60.83
W0.5 II 46	27.8	29.2	40.0	76.7	46.8	44	38.8	76.36
W0.5 I 8	25.0	33.3	71.4	77.8	58.4	45.4	44.0	59.23
W 1 I 59	26.2	43.8	61.9	61.7	54.6	45.4	48.0	48.95
W 1 II 31	14.3	29.2	45.2	40.5	53.2	48.2	46.0	33.12
W 1 II 19	16.7	20.4	46.3	46.7	58.4	48.2	52.0	38.86
W 1 II 25	8.3	27.8	60.4	61.1	45	49.6	38.0	96.53
W 1 II 75	14.8	29.2	48.1	78.6	54.4	51	41.2	67.63
W 1 I 3	16.7	25.0	40.5	70.0	50.4	51	38.0	53.05
W 1 II 148	24.1	22.9	30.6	33.3	52.6	55	38.0	28.84
W 2 II 35	31.5	31.3	57.1	62.5	48.4	52	38.0	51.68
W 2 II 37	13.0	64.6	83.3	80.0	43	44	35.2	-
W 2 I 62	16.7	25.9	61.1	81.3	61.4	46.2	35.2	77.32
W 2 I 61	13.0	33.3	46.7	73.3	43	44	31.0	66.13
W 2 I 52	24.1	27.8	46.3	52.4	59	49.6	38.8	57.05
W 2 I 7	22.9	28.6	45.2	46.7	43	51	42.0	56.27
W 2 II 29	33.3	28.6	64.3	78.6	59	46.8	38.8	59.82
W 4 II 25	20.8	40.7	33.3	42.9	48	55	38.0	36.04
W 4 II 7	20.4	33.3	60.0	79.2	47.6	49.6	38.0	55.19
W 4 II 25	-	36.7	43.8	46.7	52.2	54.2	48.0	40.55
W 8 II 71	22.2	20.8	68.2	83.3	47	49.6	33.8	46.97
W 8 II 13	12.5	27.1	43.8	40.5	57.6	49.6	46.0	33.66
W 16 II 11	14.8	29.6	62.5	70.0	54.4	48.2	36.6	50.88
W 16 II 74	14.6	16.7	35.0	46.3	56.8	53.4	52.0	46.15
W 16 I 45	25.0	33.3	47.6	30.6	45.8	49.6	38.0	37.63
W 16 I 50	27.1	19.0	53.3	80.0	46	44	32.4	57.60
W 16 I 14	20.8	22.9	55.6	90.0	50.4	44	45.2	38.74
W 32 I 82	28.3	16.7	56.3	72.2	56.8	44	37.4	94.20
W 32 II 20	16.7	37.5	47.6	69.4	43	49.6	38.0	78.25
W 64 I 100	27.1	31.3	56.3	86.1	56.2	44	36.6	70.63
W 64 I 37	20.4	38.9	45.2	38.1	55.4	48.2	38.0	34.88
W 64 I 97	-	22.2	54.8	60.0	43	44	38.0	-
W 128 II 27	16.7	37.0	52.8	73.3	49.4	46.8	38.8	96.12
W 128 II 44	25.0	20.4	41.7	42.6	50	48.2	41.2	34.81
W 128 I 55	18.8	25.9	45.8	41.7	58.4	45.4	49.2	-
W 128 II 29	18.5	27.8	59.5	66.7	57.6	51	38.0	62.15
W 128 I 67	13.9	18.8	39.6	38.9	60.6	48.2	48.0	36.02
W 128 II 1	23.8	22.2	52.1	73.8	58.4	46.8	38.0	43.96

Table 12. DDP-days, II-% and LDNA-% of selected candidates in 'Williams' and 'Cavendish Enano'.

Candidate code No.	DDP-days	II-%	LDNA-%
W 1 II 19	52.9	32.5	38.9
W 1 II 31	49.1	32.3	33.1
W 1 II 148	48.5	27.7	28.8
W 8 II 13	51.1	31.0	33.7
W 16 II 74	54.1	28.1	46.2
W 128 I 67	52.3	27.8	36.0
CE 4 II 30	53.6	31.0	40.7
CE 64 I 5	53.5	30.4	46.9

Letter in code number means W = 'Williams' and CE = 'Cavendish Enano'.

Table 13. Factor of effectiveness and putative mutations by carbon ion-beams observed in 'Cavendish Enano'.

Mutagen	No. of total plants	No. plants with variation	Characteristics	Gy dose inducing mutation (*n*)	Factor of effectiveness (%)
Carbon ion beam	42	2	Good response against to black Sigatoka	4 (1) 64 (1)	2.38 2.38
Carbon ion beam	85	1	Fast growth plantlet	4 (1)	1.17
Carbon ion beam	29	5	Hexaploid	4 (5)	17.24

Table 14. Factor of effectiveness and putative mutations by carbon ion-beams observed in ‘Williams’.

Mutagen	No. of total plants	No. plants with variation	Characteristics	Gy dose inducing mutation (*n*)	Factor of effectiveness (%)
Carbon ion beam	40	6	Good response against to black Sigatoka	1 (3) 8 (1) 16 (1) 128 (1)	7.5 2.5 2.5 2.5
Carbon ion beam	84	1	Hexaploid	4 (1)	1.69

Chapter 5

General Discussion

Gamma rays and Carbon ion-beams irradiation technique were applied to banana explants. The both methods acted different when banana explants were subjected. The biological effects of both Gamma rays and Carbon ion-beams irradiation were clearly elucidated as is reflected on the survival rate of the explants using four cultivars of banana. Gamma rays and Carbon ion-beams produce the same tendency of survival rate, mean that increasing dosage also increased mortality, but both methods are different in terms of linear energy transfer. Hase *et al*. (2004) mentioned that it is well known that high-linear energy transfer (LET) radiation such as heavy ion have higher biological effects than low-LET radiation such as gamma rays and X-rays. There is much evidence that ion beams with a LET of 100-200 keVμm^{-1} have the maximum effect, demonstrating that ion beams are more effective than the low LET radiation.

When Gamma rays was applied to the 'FHIA-01', high tolerance to the irradiation even at the doses 300-500 Gy was shown, in contrast 'Cavendish Enano', 'Williams' and 'Orito' were affected drastically by these dosage. On the other hand, when Carbon ion-beams were used in the four cultivars, 'Cavendish Enano' and 'Williams' were affected severely at the doses of 16, 32, 64 and 128 Gy. However; 'FHIA-01' and 'Orito' that even were not irradiated with the doses of 32-128, but up to 16 Gy the tendency was to produce high mortality. Regarding to the LD_{50} both cultivars 'FHIA-01' and 'Orito' show evidently high sensitivity to both Gamma rays and Carbon ion-beams, but in each irradiation method the Gy range were different.

Concerning to the weight and height of plantlets on the ‘Cavendish Enano’, ‘Williams’, ‘FHIA-01’ and ‘Orito’ using Gamma rays and ‘Cavendish Enano’ and ‘Williams’ with the first irradiation of Carbon ion beams up to 16 Gy, the tendency was to decrease weight and height when the irradiation was increased. When second Carbon ion beam irradiation was applied, the explants behave different in cluster’s height and weight. ‘Williams’ which was the one irradiated twice by Carbon ion-beams tend to remind similar in cluster’s height and weight up to 8 Gy. The same pattern showed ‘Orito’ and ‘FHIA-01’.

According to the results obtained by Gamma rays in the “Gamma room”, the survival rate of ‘FHIA-01’ was higher than the other cultivars and was even able to sustain dosages of up to 500 Gy but with diminishing survival rate. This result suggests that ‘FHIA-01’ can be used in future trials utilizing doses between 150 and 500 Gy. In addition, The flow cytometer analysis reported difference of relative DNA content only in ‘FHIA-01’ when 200 and 300 Gy were applied, suggesting that DNA deletion occurred, being definitely a promising cultivar with high sensitivity response to Gy exposure with a high chance of improving its quality by mutation induction. Seventeen plant of ‘FHIA-01’ were selected with diminished relative DNA content and growth at greenhouse conditions. An interesting sigmoid drooping leaf plant mutation of ‘FHIA-01’ have been occurred. The characteristics have been observed in the first and second generations (mother plant and sucker) suggesting that this characteristic is heritable to the subsequent generations. A sigmoid drooping leaf is a peculiar characteristic in certain *Musa* cultivars such as ‘FHIA-03’, which is a cooking banana (AABB) with high production and good vigor (Reyes-Borja, 1995). Unfortunately, bunch characteristics of

FHIA-01 were not able to compare due to chilling injury faced by the plants in the greenhouse on January, 2006.

On the other hand, when relative DNA content was analyzed on irradiated plants by Carbon ion-beams, hexaploids cells were reported, suggesting that chromosome duplication have been occurred. Yu (2006) have explained that a chromosome functions according to its basic unit - the gene. Thus, in the final analysis, genetic variations stem from changes in chromosomes and genes, commonly called “mutation”. Genetic substances are situated on the chromosome in a significant sequence. Hence a chromosome is actually a complex higher-class cell organelle, which is the core of cell division activity and provides for growth, development and reproduction with all the necessary information for replication. Normally, the karyotype of various biological chromosomes is stable. Only when the structural formation and number of chromosomes change the organism will suffer from damage and even death or loss of fertility. From a molecular point of view, mutations are changes occurring inside genes due to increase or decrease or transition or transversion of one to several nucleotides. Aberrations of chromosomes are macro-structural changes, normally meaning changes in the structure, behavior or number of chromosomes. Chromosome aberrations and gene mutations are related each other and cannot be completely separated. Because the chromosome is the gene carrier, any change in chromosome structure or number will he associated with gene changes. On the other hand, a certain gene point on the chromosome changes and then the chromosome structure will be different from the original. In nature, chromosome aberrations can be caused by some factors due to temperature, cosmic radiation or disease. When physical and chemical factors are utilized to treat cells artificially, the rate of chromosome aberration can be greatly increased. As seen from the primary

process, after the chromosome suffers damage due to physical and chemical factors, structural changes might result, if the damaged structure recovers, there will be no change in function. Thus, chromosome damage is a prelude to variations.

On the topic of the banana plants subjected to the Gamma rays in “Gamma Field” did not confer any visible abnormalities during the 34-day exposure, in contrast with the materials irradiated in the “Gamma Greenhouse” visible variation was shown 9 months after irradiation such as abnormal, double, long, rudimentary, spindled and yellow spotted leaf, caused by the effect of the long-term chronic irradiation which acts directly in the active cell division occurred in the meristem. These results suggest that an irradiation scheme using different exposure times could be explored to avoid injuries at the meristem levels.

Interesting results were also obtained when the irradiated plants from Gamma rays and Carbon ion-beams were subjected to juglone. From Gamma rays irradiated materials, 20 plants were selected from the Orito lot, 8 in ‘Williams’ and 5 in ‘Cavendish Enano’. From Carbon ion-beams irradiated plants 10 plants showing lower values of leaf necrotic area were selected. However, these results are not yet final, as further studies need to be implemented to confirm the tolerance to juglone in correlation with the results of the materials subjected to natural inoculum pressure of *M. fijiensis* under field condition.

The three variables DDP-days, II-% and LDNA-% were combined analyzing the data by regression, permitting to categorize the plants showing better response against to the disease. The regression among the three combined variables showed close relationships in six plants. The code number were ‘W 16 II 74’, ‘W 128 I 67’, ‘W 1 II 148’, ‘W 8 II 13’, ‘W 1 II 19’ and

‘W 1 II 31’. In the case of ‘Cavendish Enano’, two plants with the code numbers ‘CE 4 II 30’ and ‘CE 64 I 5’ were observed. These groups of plants were selected as possible candidates with better response against to black Sigatoka. Those results allow to allege that the sensitivity to the irradiation of ‘Williams’ determined by LD_{50} could explain the variation in the number of mutated plants that shows six candidates, compared with the two candidates obtained in ‘Cavendish Enano’. That means that high sensitivity cultivars to Carbon ion-beams irradiation may produce a wide range of mutagenesis expressing tolerant/resistant to black Sigatoka, deducing that the sensitivity is cultivar dependent.

A single plantlet of ‘Cavendish Enano’ with early growth has been also observed among a group of plants irradiated by Carbon ion-beam at 4 Gy (Fig. 49). This characteristic is important for crop cycle; meaning that the annual production could increase due to the number of bunches harvest per year.

In conclusion, Gamma rays and Carbon ion-beams produce the same tendency of survival rate when banana explants were subjected to their irradiation, but both methods are different in terms of linear energy transfer, showing the effectiveness of Carbon ion-beams at low doses. The most sensitive to the irradiation among the banana used in this experiment were ‘Orito’ and ‘FHIA-01’. A sigmoid drooping leaf plant of ‘FHIA-01’ and plants with low incidence affected by black Sigatoka and juglone toxin were obtained. In addition, a single plant of ‘Cavendish Enano’, irradiated by Carbon ion-beam at greenhouse conditions showed the early growth among a group of plants. Regarding to the selected plants less affected by black Sigatoka, field experiment considering the whole plant cycle must be necessary to confirm not only the black Sigatoka response but also fruit

quality and potential production and post harvest evaluations as necessary components for final selections. Additionally, mutant plants such as sigmoid drooping leaf, fast growth, hexaploids and diminished DNA content plants also should be necessary to carry on studies on field condition to confirm if the new traits are single or linked genes.

Summary

Both Gamma rays and Carbon ion-beams irradiation methods were applied in order to identify the critical irradiation doses, to induce genetic variability for mutant selection and to evaluate the response of black Sigatoka disease. Both irradiation methods acted different in terms of biological effect when banana explants were exposed. Gamma rays and Carbon ion-beams produced the same tendency of survival rate, meaning that increasing dosage also increased mortality, but both methods were different in terms of linear energy transfer. 'FHIA-01' tolerated high doses of Gamma rays, but was susceptible to the high doses of Carbon ion-beams. The Gamma rays results suggested that 'FHIA-01' can be explored using other dose intervals among 150 Gy and 500 Gy. Weight and height of plantlets were affected by Gamma rays and Carbon ion-beams. Gamma rays reduced drastically these characters when doses of 200-300 Gy were applied. Carbon ion-beams also affected the height and weight with the doses between 4 and16 Gy. LD_{50} of both cultivars 'FHIA-01' and 'Orito' manifested high sensitivity to both Gamma rays and Carbon ion-beams, although in each irradiation method the Gy energy were different.

During the second Carbon ion beam irradiation the explants behaved different. Weight and height remained stable up to 8 Gy and the survival rate was reduced much more between 8 to 16 Gy.

The relative DNA content of 'FHIA-01' was reduced by Gamma rays at 200 and 300 Gy, suggesting DNA deletion. 'FHIA-01' is definitely a promising cultivar with high sensitivity response to Gy exposure with a high chance of improving its quality by mutation induction. Sigmoid drooping leaf, a putative mutation of 'FHIA-01' was generated. As first and second

generation (mother plant and sucker) showed the same characteristics, this characteristic is heritable to the subsequent generations. Unfortunately, bunch characteristics and fruit quality were not able to be compared, but future research could be conducted for leaf shape in relation to production and fruit quality. On the other hand, Carbon ion-beams irradiated plants showed hexaploids cells detected by flow cytometer (five plants in ‘C. Enano’ and one in ‘Williams’), signifying that chromosome duplication occurred.

On the topic of Gamma rays irradiated plants in “Gamma Greenhouse”, visible variation of the leaves such as abnormal, double, long, rudimentary, spindled and yellow spotted leaf were shown, suggesting that the effect of the long-term chronic irradiation acted directly in the active cell division at meristem level, resulting in severe damage or even death of the meristems.

A screening method using juglone toxin was adapted using the leaf disc from young banana irradiated plantlets. Leaf disc necrotic area (LDNA-%) was determined selecting the less affected plants form the irradiated population (Gamma rays). Twenty plants were selected from the ‘Orito’ lot, eight in ‘Williams’ and five in ‘Cavendish Enano’.

Applying a lineal regression to the combinations of the disease development period (DDP-days), infection index (II-%) and LDNA-% on plants from ion beam experiment, six plants of ‘Williams’ (code number: ‘W 16 II 74’, ‘W 128 I 67’, ‘W 1 II 148’, ‘W 8 II 13’, ‘W 1 II 19’ and ‘W 1 II 31’) and two plants of ‘Cavendish Enano’ (code numbers: ‘CE 4 II 30’ and ‘CE 64 I 5’) were selected as possible candidates with better response against to black Sigatoka.

A ‘Cavendish Enano’ plant with fast growing by Carbon ion-beam (4 Gy) was observed. The earliness is thought as an important characteristic for crop cycle and will result biannual.

Finally, field experiment considering the whole plant cycle must be necessary to perform, to study the relationship of the new mutant plants such as better response against to black Sigatoka, sigmoid drooping leaf, fast growth, hexaploids and diminished DNA content plants with genes of fruit quality, potential production and post harvest characteristics as components for final selections.

ACKNOWLEDGEMENT

My entire gratitude to my academic adviser, Dr. Hiroshi Gemma, Professor of the Institute of Agriculture and Forestry, University of Tsukuba for his essential supervision, guidance, availability, assistance and efficient help during my research; to Dr. Yoritoshi Okuno, Professor of the Institute of Agriculture and Forestry, University of Tsukuba for his critical review and commentary on the manuscript and Dr. Toshiya Yamamoto, Professor of the University of Tsukuba, Doctor Program of Advanced Agriculture Technology and Sciences for his valuable corrections and suggestions.

I would like to thanks to Dr. Sumiko Sugaya, Assistant Professor of the Laboratory of Pomology of the Institute of Agriculture and Forest, University of Tsukuba for her outstanding guidance and support of my research and Dr. Yoshihiko Sekozawa, Laboratory of Pomology of the Institute of Agriculture and Forest, University of Tsukuba for his support of my research.

My appreciation to Dr. Shigeki Nagatomi, former Head of the National Institute of Radiation Breeding, for his technical advice and assistance on the establishment of the experiments on Gamma ray irradiation, providing necessary facilities, which made this research possible.

Thankfulness to Dr. Atsushi Tanaka, Head of the Institute of Takasaki Ion Accelerators for Advanced Radiation Application (TIARA), Japan Atomic Energy Agency (JAEA), Japan, for is collaboration providing necessary facilities to make this research possible and Dr. Yoshihiro Hase, Researcher of the (TIARA-JAEA), Japan, for his valuable orientation on irradiation for better conduction of this research

I extend my gratitude also to Dr. Kazuo Watanabe, Professor of the University of Tsukuba, Doctor Program of Bio-Industrial Sciences for his technical assistance during flow cytometer and viruses analysis.

Thanks to M. Sc. Ingnacio Sotomayor Herrera, Director of the Estación Experimental Tropical Pichilingue (EETP), Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), for his disinterested collaboration providing facilities for the normal conductance of the field experiments, to M. Sc. Iván Garzón, Head of the Laboratory of Biotechnology, Estación Experimental Tropical Pichilingue (EETP), Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), for his support for evaluations of the banana materials at nursery and field conditions.

I am appreciative to Dr. Carmen Suarez, M. Sc. Danilo Vera and Egdo. Roy Vera, Laboratory of Phytopathology, Estación Experimental Tropical Pichilingue (EETP), Instituto Nacional Autónomo de Investigaciones Agropecuarias (INIAP), providing *M. fijiensis* colonies for a better conductance of this project and Ing. Mariuxi Cedeño, Tcnga. Behtty Castillo, Mr. Roberto Loza, Laboratory of Biotechnology (EETP-INIAP), for their valuable collaboration for evaluations at field conditions.

I am grateful for Ms. Danilda Duran Hufana for her unconditional help and editing of the manuscript and M. Sc. Luis Duicela for his support with the statistical analysis.

I wish to thank the Ministry of Education, Science and Culture of Japan for the scholarship allowing me to purse my Doctoral degree at the University of Tsukuba, Japan.

Thanks also to all those who have contributed from the start and completion of this study and whose names are not mentioned.

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