2. Mutation Breeding Technology

2.1 Mutation induction for banana improvement

2.1.1 Bangladesh

Mutation breeding is a process by which a gene undergoes a structural change or substitution of one nucleotide for another. A variant emerges, differing genetically and often visibly from its parents and arising rather suddenly or abruptly. Mutation can occur naturally or can be induced by radiation or chemically.

Banana is the world's major food crops and considered as the poor man's fruit in tropical and sub-tropical countries. Edible bananas are mostly sterile polyploids and must be propagated vegetatively. Hence, genetic improvement through cross breeding is not possible. Mutation breeding has been suggested as an excellent alternative approach for banana improvement (Guzman, 1975). Genetic variation is the starting point of any breeding program. Genetic variation may already be present in nature, may be obtained after several years of selection or may be produced through hybridization. For vegetatively propagated crops such as edible banana, it is difficult to obtain genetic variation, because of sterility and polyploidy nature of the crop. Spontaneous somatic mutants have played an essential role in the speciation and domestication of plantain and banana. All bananas and plantains that we grow and eat were selected in prehistory from spontaneous mutations (Buddenhangen, 1987). Radiation can be used to induce mutations at a higher frequency and generate genetic variation from which desired mutants may be selected. Therefore, mutation techniques are important as tools for plantain and banana improvement. Still there is a lot of scope to yield improvement of banana for enhanced food security, nutrition and employment generation. Progress in the development of various biotechnologies has greatly contributed to the application of induced mutations in a wide range of plant species. This is new opportunities to induce mutation in vegetatively propagated crops by the use of *in vitro* techniques. The large scale rapid multiplication of the resulting mutants and characterization using molecular markers is necessary to understand gene structure and gene function. Generally, radiation and especially gamma rays have most often been used to generate desired characters for crop breeding. This technique can be used in many crop species, often those with narrow genetic variation, to improve individual or specific characters in local cultivars that are particularly adapted to specific environmental conditions. Induced mutation techniques are particularly important for banana and plantain where there is limited sexual reproduction that could generate genetic variation. Several researchers suggested that mutagenic agents for induction of resistance to several diseases using regenerating adventitious buds of banana are effective (Panton and Menedez, 1972; Menedez, 1973).

2.1.2 Malaysia

Cultivated bananas are *parthenocarpic*, which makes them sterile and unable to produce viable seeds. Propagation typically involves removing and transplanting part of the underground stem (called a corm). Usually this is done by carefully removing a sucker (a vertical shoot that develops from the base of the banana pseudostem) with some roots intact. It is not necessary to include the corm or root structure to propagate bananas; severed suckers without root material can be propagated in damp sand, although this takes somewhat longer. Within the last 15 years, commercial propagation occurs by means of tissue culture. This method is preferred since it ensures disease-free planting material. When using vegetative parts such as suckers for propagation, there is a risk of transmitting diseases, especially Fusarium wilt, Black Sigatoka and also nematodes.

Most edible bananas are considered to be sterile, therefore genetic improvement through cross-breeding is not possible. Mutation breeding through vegetative propagation has been suggested as an excellent alternative approach for banana improvement (Valez et al., 1972, Siti Hawa et al., 1996). In addition, the heterozygosity of asexual banana clones makes them suitable for mutation induction. The heterozygotic status is expected to be Aa in loci of diploid cultivars while the triploids of A genomic types can exist in either Aaa or Aaa forms. For interspecific hybrids, the heterozygotic constitution could be AaB, Aab, AAb, ABb, aBb or aBB. Mutation induction may uncover a recessive phenotype by mutating, inhibiting or deleting the corresponding dominant allele (Novak et al. 1990).

Mutation is a single cell event and one advantage to utilize mutation breeding is that a reduced breeding time can be achieved. In a conventional cross-breeding program, if a useful trait is to be transferred into an economically important cultivar, this will necessitate the crossing of the economically important cultivar with perhaps a less desirable variety possessing a single advantageous agronomic trait. It will then be necessary to take the F_1 hybrid and backcross it to the superior parent cultivar for at least 7-8 generations, with continued selection of individuals possessing the particular useful trait. In a mutation breeding program, the same breeding objective may be able to induce a mutation of the desired trait in the economically important cultivar. If the trait is successfully obtained in the M_2 generation, purification of the mutant requires only 1 or 2 additional generations. In a mutation breeding program it is important to understand the mode of reproduction of the crops (sexual or asexual, self-pollinated or cross-pollinated). In addition, especially in banana, understanding how the ploidy level (diploid or polyploid) may affect trait expression is also an important consideration.

The use of cultured shoot tips for mutagenesis has facilitated mutation induction and the regeneration of potential mutants (Novak and Micke, 1990). An early flowering mutant of Grand Naine, GN-60Gy was induced by Novak (1990) after exposing shoot tips to gamma radiation. Further selection of GN-60Gy in Malaysia has resulted in the release of an early fruiting Cavendish banana called Novaria (Mak Chai et al., 1996).

2.1.3 Philippines

The germplasm of commercially important banana cultivars, both dessert (AA, AAA) and cooking (ABB, BBB) bananas, are not amenable to sexual breeding because they are male and female sterile. Thus, BBTV resistance could not be introgressed into bananas by conventional breeding methods. In addition, there is no known resistance to BBTV in the banana germplasm. In other crops, it has been demonstrated that it is possible to obtain resistance to pests and diseases by variation brought about by irradiation, chemical mutagens, somaclonal variation or by a more direct method of *in vitro* selection in the presence of the stress factor (Roux, 2004).

Mutation and *in vitro* technologies offer opportunities to enhance genetic variability for the improvement of agronomic traits such as disease resistance, earliness in fruiting, yield and quality (Bhagwat and Duncan, 1998; Ho *et al*., 1994; Mak *et al*., 1996; Novak *et al*., 1993; Roux, 2004; Smith *et al*., 1995). In addition, the availability of tissue culture techniques aids in the induction, selection and multiplication of mutants. Plant regeneration from banana shoot tips (Damasco and Barba, 1984; Damasco *et al*., 1984) is well established.

2.1.4 Vietnam

The national banana network consists of many research institutions, laboratory and agricultural cooperative conducting different activities in their area of expertise as The Vietnam Agricultural Science Institute (VASI), the National Repository, Multiplication and Distribution Centre, is incharge of conservation of *in-vitro* collection, its propagation and distribution for evaluation, testing and field trials. The Southern Fruit Research Institute (SOFRI) conducts activities such as maintenance of a field collection, disease indexing, production of disease-free planting materials, field trials and postharvest technology. The Fruit and Vegetable Institute established a procedure of banana micro-propagation, provides *in-vitro* plantlets to farmers, and implements trial for *Fusarium* evaluation. The Institute of Agricultural Genetics (AGI) applies mutation technique in plant breeding and molecular technique (RAPD, PCR etc.) in studying banana biodiversity and virus indexing. Fusarium wilt is the second important diseases of banana. It attacked Chuoi Tay (Pissang awak-ABB), Chuoi Ngop (Bluggoe ABB), and huoi Com La (Silk-AAB). Average frequency of infected plants in Chuoi Tay cultivar in summer (24.3%) was higher than in winter (13.2%) in north Vietnam. In the regions which Chuoi Tay was grown much, frequency of infected plant was increased (Table 2). They may cause up to 85% yield loss in banana. So that we focused on application of mutation technique in generate a new mutant local banana for resistance to the disease by Foc.

2.2 Selection of target banana cultivars

2.2.1 Bangladesh

The popular dessert banana in Bangladesh is Sabri (AAB) but the yield is not satisfactory. Moreover, the second leading commercial cultivar Sabri (AAB) is highly susceptible to Panama disease (Fusarium wilt) and total crop failure due to this disease has been reported. The cultivar also can not tolerate water logging condition. These are the main reasons to choose this cultivar to overcome those problems. A local seeded cultivar 'Bichikala (BB)' derived from the wild species of *Musa balbisiana* (BB) is resistant to most of the diseases and pests and also has some degree of tolerant to draught and water logging condition. In addition, it is sweeter than that of seedless popular banana cultivar produced in the country and it has much more medicinal value as well. It has also high keeping quality and widely grown in the country in homestead area. But the main constraint of the fruit is that it contains huge number of seeds that makes them less popular to the local people. Therefore, this cultivar is chosen to create genetic variations such as seedless or less seeds in the fruits with improve agronomic traits through the production of doubled haploids. The cultivar Sabri is seedless table fruit and belongs to the genome (AAB). The plant is 2.5 to 3.0 meter tall. It takes 8 to 9 months for flowering after plantation of sucker and another 3 to 4 months needed to ripening the fruit. The total bunch weight is about 9 to 10 kg, in which 7 to 10 hands/bunch and 10 to 12 fingers/hand. Per hectare yield is 14 to 15 tones. The fruit peel became bright yellow when ripen and the peel is very thin. The fruit pulp is off-white in colour. The cultivar Bichikala is a seeded banana eaten as vegetable before ripen and also eaten as a table fruit when ripen and belongs to the genome (BB). The number of seed per fruit is about 150 to 250 and the seeds are soft and brownish in colour. The plant is very strong and tall of about 4.5 to 6.5 meter. It takes 10 to 12 months for flowering after plantation of sucker and another 4 to 5 months needed to ripening the fruit. The total bunch weight is about 15 to 20 kg, in which 10 to 15 hands/ bunch and 9 to 12 fingers/hand. Per hectare yield is about 20 to 25 tones although this cultivar is not producing commercially. The fruit peel became yellowish when ripen and fruit peel is very thick. The fruit pulp is off-white in colour.

2.2.2 Malaysia

The popular dessert cultivars in Malaysia are Mas (AA), Berangan (AAA), Rastali (AAB), Embun (AAA) and Cavendish (AAA). However, Pisang Berangan (AAA) is the most popular banana, having good fruit quality, flavour, colour, pulp texture, size and shelf life. About 50% of the banana growing land is cultivated with Pisang Berangan and the Cavendish type for local consumption and export market to Singapore. However, it is relatively tall and very susceptible to Fusarium wilt (*Fusarium oxysporium* f. sp *cubense*) and freckle disease caused by *Cladosporium musae*. Therefore, Pisang Berangan was selected as the target banana cultivar for mutation induction study to select potential mutants with improved traits such as tolerance or resistance to *Fusarium* wilt disease, short plant stature and early fruiting and high bunch weight.

2.2.3 Philippines

The banana cultivar Lakatan (AA) was used as the initial material for mutation induction**.** The cv Lakatan is the most popular dessert banana grown for domestic market and as novelty banana for export market. Lakatan is very susceptible to BBTV. The Lakatan industry in some regions of the Philippines was wiped out due BBTV.

2.2.4 Vietnam

In vitro single or multiple shoots of banana are established by culturing isolated shoot tips on MS medium (Murashige and Skoog 1962) supplemented with 30 g/l sucrose and 0.5 - 4 mg/l BA (6-benzylaminopurine). For rooting of shoots, the well grown shoots were separated and transferred to MS medium containing the same concentration of BAP (3.0 mg/l) and NAA (0.2 mg/l). The pH of the medium was adjusted to 5.8 before autoclaving and has maintained at 24 ± 1 °C under 16h cool white, fluorescent lights (4,000 lux). *In vitro* shoot tips 2 - 3 leaf primordia is initial material for gamma treatment. The experiment carried out 5 formulas with five gamma doses at 10, 20, 30, 40 and 50 Gy. The results showed an appropriate gamma dose is 15 or 20 Gy.

2.3 Preparation of plant materials for irradiation

2.3.1 Bangladesh

The suckers of Sabri (AAB) chopped off 3 - 5 cm long and washed thoroughly under running tap water (Fig. 2-3-B-1a to 1c). Explants were prepared by removing the outer layer of leaf-sheaths from the suckers using a clean knife. The pale white tissue blocks of 1.5 x 2.5 cm^2 in size containing shoot tips were washed under running tap water with 'Tween 20' for 30 minutes. Then they were immersed in 80% v/v alcohol for 30 second to 1 minute followed by sterilization in 50 to 100% clorox with 2 to 3 drops of 'Tween 20' for 20 minutes, and finally rinsed three times with sterile distilled water in the laminar air flow cabinet. The ensheathing leaf-sheaths which surrounded and protected the apex, were removed to obtain shoot tips of about 0.5 cm in length with a 4 to 5 layers of leaf primordia. After surface sterilization, shoot tips of about 0.5 cm in size were treated with 8 doses of gamma rays viz., 10, 20, 30, 40, 50, 60, 70 and 80 Gy from ^{60}Co gamma irradiator at a dose rate of 25 Gy/min (Fig. 2-3-B-1d). Number of explants used were 120, 190, 104, 196, 192, 184, 170, 180 and 165 for control. Immediately after irradiation, explants were placed onto MS medium fortified with 5.0mg/l BA + 0.2% Ads (Fig. 2-3-B-1e to 1f). Radiation sensitivity and post-irradiation recovery were assessed by measuring the survival and propagation rate 40 days after irradiation. For bulk irradiation of cv. Sabri, suckers were collected from Narshingdi, one of the major banana growing districts, which is about 50 km away from Dhaka City. All together about 1,200 suckers in several batches (100 - 180 Suckers per batch) were collected from Narshingdi. Three repeated subcultures of growing shoots was carried out at one month interval on the same medium following to M_1V_4 to dissociate chimeras in the regenerated shoots (Fig. 2-3-B-1g) and were also three repeated subcultures done for control plantlets (Fig. 2-3-B-1h).

Fig. 2-3-B-1 *In vitro* mutagenesis for developing Fusarium wilt resistance in commercial cultivar 'Sabri'

a. Some collected suckers of Sabri (AAB), **b.** Chopping of suckers,
c. Surface sterilization of chopped suckers with 0.2% Bavestin (fungicide), **d.** Irradiation of suckers,
e. Slicing of shoot tips, **f.** Inoculation of shoot tip explants on culture medium,
g. Multiple shoot formation (M_1V_4) from irradiated shoot tip explants,
h. Multiple shoot formation from un-irradiated (control) shoot tip explants, **i.** Rooting of M_1V_4 shoots,
j&k. Hardening of M_1V_4 plants, l&m. Screening of M_1V_4 plants against Fusarium wilt under greenhouse condition

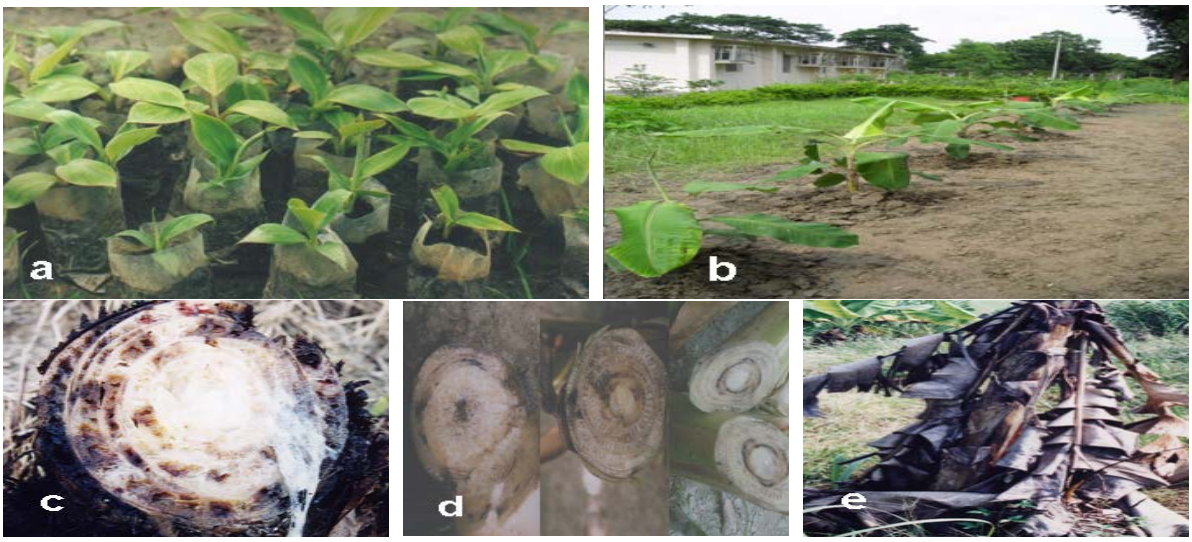


Fig. 2-3-B-2 *Fusarium* infestation in commercial cultivar 'Sabri (AAB)'
a. Irradiated plantlets in poly bags with hot spot soil, **b.** Irradiated plants in hot spot affected field,
c & d. Vascular dis-colouration and pseudo stem rottening, **e.** Death plant

2.3.2 Malaysia

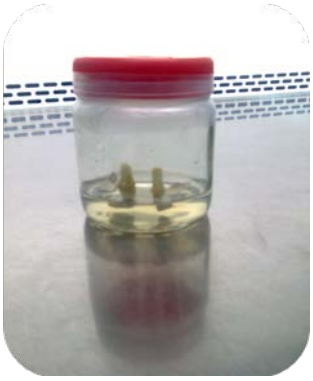
Suckers of cultivar Berangan were obtained from our collaborator, United Plantations Berhad and they were cleaned and surface sterilized using sodium hypochlorite for 30 min. They were excised into small pieces of meristem tissues about 1 - 2cm in size and explanted onto MS media containing 5 mg/l BAP. Adventitious buds begin to appear after a period of 1 - 2 months in culture and later *in vitro* shoots start to produce. Newly formed *in vitro* shoots were transferred onto fresh MS media and multiplication was routinely carried out every 3 - 4 weeks after culture. Rooting of *in vitro* shoots was established in MS rooting media supplemented with 1 % activated charcoal.

<u>Preparation of plant materials for irradiation</u>

Original suckers cleaned with tap water

Surface sterilized with Chlorox

Cutting under laminar flow cabinet



Meristem cultures for irradiation

Adventitious buds formation after 1 month

Shoots formation after 2 months

2.3.3 Philippines

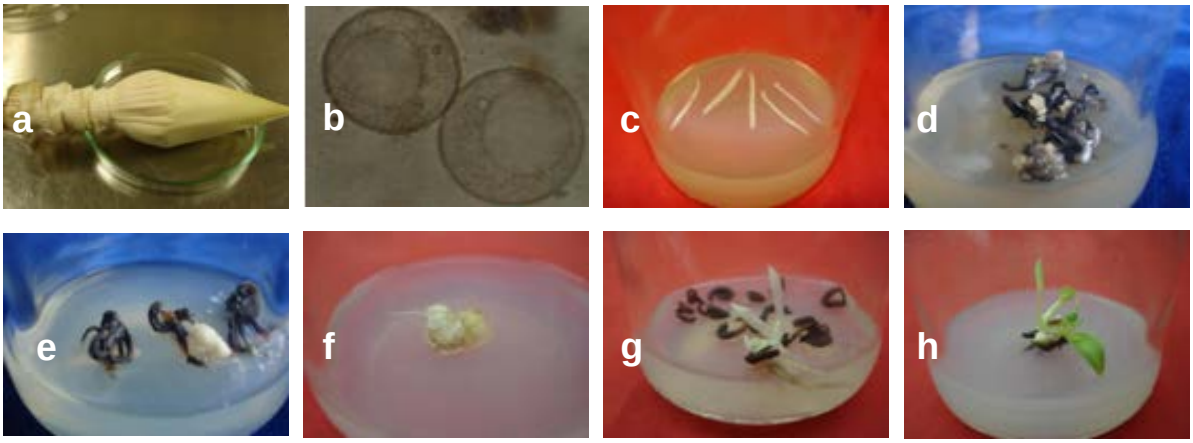
Disease free suckers of cv Lakatan collected from the field were established *in vitro* following the standard banana tissue culture procedure (Damasco and Barba, 1984). Suckers were cleaned and surface sterilized in pure bleach for 45 min. Shoot explants (1 cm x 1 cm) were excised, cut into 4 sections and inoculated onto MS medium + 5 mg/l BAP. Shoots were regularly sub-cultured every 4 to 6 weeks onto shoot multiplication medium (SMM) containing MS basal medium + 3 mg/l BAP. Shoots were transferred onto MS basal medium + 0.1 mg/l activated charcoal for rooting and plantlet development. All irradiated shoots cultures and selected mutant lines were micropropagated using the SMM.

2.4 Special culture methods

2.4.1 Bangladesh

Anther culture of diploid banana

A local diploid seeded cultivar Bichikala (BB) derived from the diploid wild species *Musa balbisiana* (BB) was used in this study. The male flower bud containing all developmental stage of pollen was used as donor material for anther isolation. The mature male flowers along with the bracts were removed from upper part of the male flower bud to get immature male flower having fully developed anthers with appropriate developmental stage of pollen. Male flower bud (Fig. 2-4-B-1a) was then surface sterilized by swabbing with cotton soaked in 70% ethanol. The bract was then separated from immature flowers and cluster of male flowers with fully developed anthers were isolated and transferred into petri dish under laminar air flow cabinet. The surrounding tepals and ovary were removed. One anther from each cluster of male flower was examined under microscope to identify the developmental stage of pollen i.e., uni-nucleate stage (Fig. 2-4-B-1b). Five anthers (Fig. 2-4-B-1c) containing uni-nucleate pollen were placed in each jar containing agar-gelled (8% w/v) MS (Murashige and Skoog, 1962) or N6 (Chu *et al*., 1975) based medium. MS medium contained basic macro- and micro-nutrients, morel vitamins (Morel and Wetmore, 1951) and supplemented either with 2.5 mg l^{-1} 2,4-D and 1.0 mg l^{-1} kinetin (designated as MS1) or with 1 mg l^{-1} BA, 0.4 mg l^{-1} IAA and 500 mg l^{-1} casein hydrolysate (CH) (designated as MS2) while N6 medium comprised basic salts and Morel vitamins, 1 mg l^{-1} BA, 0.4 mg l^{-1} IAA and 500 mg l^{-1} CH. Sucrose at a concentration of 2.5% was used as carbon source in all cases. The pH of the medium was adjusted to 5.7 before autoclaving. Cultures were kept at 26 ± 1°C in darkness and maintained on the same medium without subculture until callus is produced. Calli thus obtained (Fig. 2-4-B-1d & 1e) were transferred to fresh medium composed of MS basic salts, Morel vitamins, 0.5 mg l^{-1} BA and 0.4 mg l^{-1} IAA and kept under fluorescent illumination (at 1,500 lux intensity) with 16/8 h light/dark cycle for regeneration of plants. Cytological study of the root tips was carried out for ploidy determination of the regenerated plants.



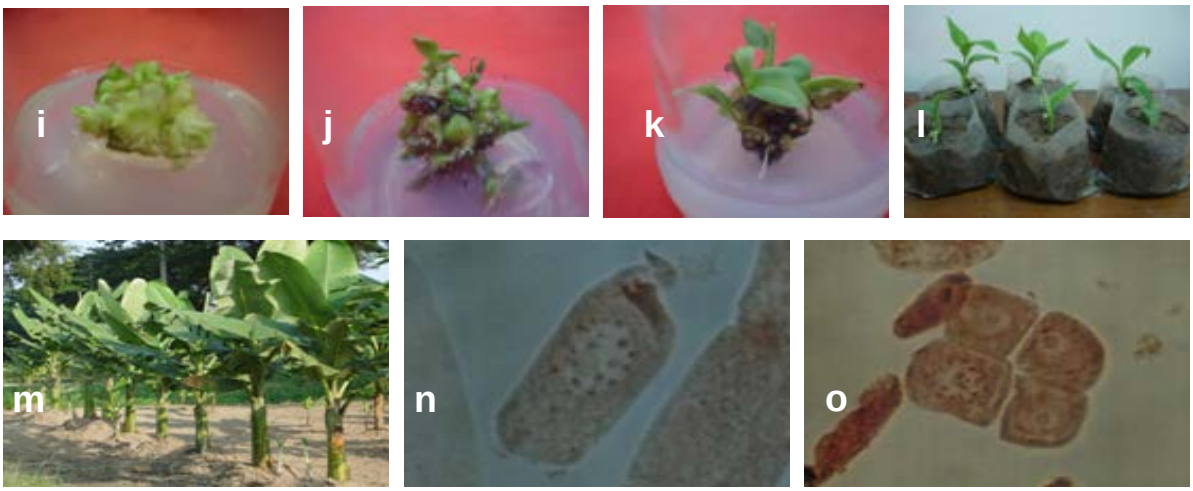


Fig. 2-4-B-1. Anther culture in *Musa balbisiana* cultivar 'Bichikala'

a. Male flower bud, **b.** Uni-nucleate pollens, **c.** Anthers on the culture medium,
d. Starchy callus on 2,4-D and kinetin supplemented MS medium,
e&f. White and compact callus on BA, IAA supplemented MS medium,
g&h. Shoot regeneration on N6 based callus induction medium, **i.** Callus on regeneration medium, **j&k.** Regeneration of shoots from calli/embryoids, **l.** Acclimatization of plantlets,
m. Anther-derived plants in the field, **n&o.** Root tip cytology for chromosome counting

2.4.2 Malaysia

Application of somatic embryogenesis for plant regeneration can be used as plant materials for mutation induction. Somatic embryos were successfully induced from male inflorescence of *Musa acuminata* var. Mas (AA). Suspension cultures were successfully initiated and translucent spheres and torpedo shaped embryos were obtained upon transfer to embryo development and regeneration medium. Complete plant regeneration from embryogenic cell suspensions were also obtained.

I. Explant source & sterilization

Inflorescence male buds of *Musa acuminata* cv. Mas (AA) were used as explant materials. First dissection was carried out in the general lab where the male bud was shortened to 6 - 8cm in length by removing the enveloping bracts. For second dissection the male bud was then transferred to a laminar air flow. Explants were sterilized in 70% ethanol for about 15 minutes and rinsed 3 times with sterile distilled water. The male bud was further shortened to 1 - 1.5cm in length for culture and immature male flower clusters position 1 - 15 were removed under stereo-microscope until the meristem is exposed.

II. Initiation of embryogenic callus

Immature male flowers were cultured on initiation medium, M1 (Escalant *et al*. 1994; Cote *et al*., 1996) to initiate embryogenic callus supplemented with combinations of different concentrations of 2,4-D over 2 subcultures for every 3 months.

III. Suspension culture

Friable embryogenic callus was transferred to two different suspension medium that is M2a (Cote *et a*l., 1996) and M2b (Dhed'a *et al*, 1991) to optimize the best medium for initiation of suspension culture for *Musa acuminata* cv. Mas (AA). Growth of cell suspensions was measured for the selected M2 media.

IV. Development of somatic embryos

Embryos were developed on M3 (Cote *et al*, 1996) media.

V. Regeneration of somatic embryo

Regeneration of somatic embryos was performed on M4 (Cote et al., 1996) medium with 3 different concentrations of BAP (0.05mg/l, 0.1mg/l and 0.2mg/l) and MS media without any plant growth regulators (MS0).

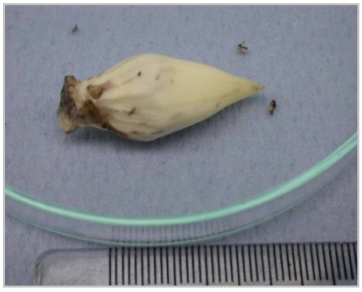
VI. Culture conditions

Cell suspensions were maintained at 70 r.p.m continuously on a gravitory shaker.

For plant regeneration, cultures were initially placed in the dark until shoot bud appeared and subsequently transferred to light conditions.

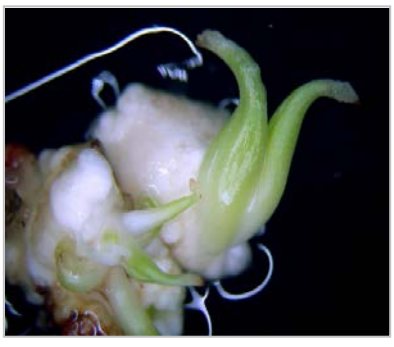
Somatic embryogenesis for plant regeneration

Male flower



Male bud as explant

Somatic embryos



Shoot regeneration

Multiplication of *in vitro* shoots

Rooted plantlets

2.4.3 Vietnam

I. Effect of gamma ray on subsequent survival of banana shoot tips cultured *in vitro*

Under the effect of radiation ray, which should be reduce the growth and morphogenetic performance to 5 - 45 % of the control. Considerable phenotypic variation was observed among the

shoots regenerated from shoot tips. In early stages of shoot development the irradiation affected emergence and expansion of the youngest leaves. The vegetative growth was influenced by a higher dose of irradiation (above 20 Gy). Here we calculated for the percentages of surviving after mutagenic treatment of shoot tips observed at 7, 14, 21, 28 days. Surviving percentage as described in Table 2-4-V-1.

Table 2-4-V-1. Surviving percentage of *in vitro* cultured shoot tips of dwarf banana

Dose of gamma irradiation (Gy)	Survival days (%)				
	1 day	7 days	14 days	21 days	28 days
0	100.0	100.0	100.0	100.0	100.0
10	100.0	95.0	84.2	82.2	79.0
20	100.0	93.8	80.4	78.6	76.4
30	100.0	87.4	63.6	57.0	52.0
40	100.0	82.2	57.0	42.6	35.6
50	100.0	77.0	34.6	26.2	22.6

Table 2-4-V-2 and Fig. 2-4-V-1 showed that: Gamma ray has direct influence to surviving of Tay banana shoot tips, if irradiation dose is increased, surviving percentage should be decrease and decreases at 30 Gy. In seven first days, surviving percentage decreases smoothly and increases significantly following 14 days of cultivation in all formulas. At that time, gamma ray made many shoots turned black in color and died. At irradiation dose 10 and 20 Gy, there weren't differences in surviving percentage. In 50 Gy, living shoot tips had phonotypic changed with yellow leaf and slow growth .There were a little of living shoot tips.

II. Effect of gamma irradiation dose on propagated coefficient of *in vitro* cultured

After 28 days of gamma treatment, banana shoot tips were transferred on medium MS supplemented with 0.5 ppm Naphthalene acetic acid (NAA), 4 ppm Benzylaminopurine (BAP). We reported on propagated coefficient (after 5 times subculture). As shown by the results given in Table 2-4-V-2. Gamma ray has direct influence to propagated coefficient of banana shoot tips; the shoot propagated coefficient is lower than control. In the 3rd of subculture, this coefficient was constant and shoots formation grown well in the cultured medium.

Table 2-4-V-2. Effect of irradiation dose on surviving percentage (5 times subculture)

Dose of gamma irradiation (Gy)	Propagated coefficient (times)				
	1st	2sd	3rd	4th	5th
0	3.22	3.27	3.83	3.72	3.72
10	1.30	1.80	2.53	2.92	3.34
20	1.19	1.57	2.12	2.51	2.81
30	1.10	1.52	1.87	2.14	2.66
40	1.08	1.29	1.32	1.38	2.03
50	1.05	1.15	1.10	1.05	1.91

III. Effect of gamma ray on percentage root initiation of banana shoots

Beside propagated coefficient, quality of roots and percentage root initiation is also target to evaluate effect of gamma ray on banana shoot tips. In this period, shoots were transferred on half-strength MS medium, supplemented with 0.05 ppm NAA, 0.02 ppm IBA. The number of roots was counted at the last internal after 7, 14, 21, 28 days.

Table 2-4-V-3. Effect of gamma irradiation dose on percentage root initiation of plantlets

Dose of gamma irradiation (Gy)	Percentage root initiation (%)				
	7 days	14 days	21 days	28 days	Quality of root
0	47.78	66.67	78.89	93.33	+++
10	14.44	58.89	72.22	88.89	+++
20	12.22	52.22	66.67	86.67	++
30	4.44	37.78	53.33	72.22	+
40	3.33	36.67	50.00	70.00	+
50	2,56.	36.10	43.00	62.00	

+: weak root; ++: average root; +++: strong root

Table 2-4-V-3 showed that the gamma ray had direct influence to appearing roots of banana shoots. Percentage root initiation decreased after mutagenic treatment and root was slow growth and weak (except 10 Gy irradiation dose). The percentage root initiation decreased smoothly in 14 first days, the lowest level in 40 Gy (36%)

IV. Effect of gamma ray on appearance variation of banana shoots

Adventitious origin of shoot buds from the superficial cells of the rhizome cultured *in vitro* reveals the possibility that entire shoot can be raised from a single somatic cell. This system is particularly important for practical mutation breeding because a high frequency of solid plant mutants can be obtained after explanting irradiation, these results described in Table 2-4-V-4.

Fig. 2-4-V-1. a. Dwarf plants, b. Thin variation, c. Color variation

Table 2-4-V-4. Effect of irradiation dose on mutation frequency

Dose of gamma irradiation (Gy)	Variation							
	Stump	Long-shoots	Dwarf-shoots	Stem color	Formed leaves	Leaf-like stem	Leaf-blade like stem	Total
10	1.7	0.1	0.1	1.5	1.5	0.1	2.5	7.5
20	2.3	0.1	0.1	3.5	3.5	0.5	4.5	14.5
30	2.3	0.1	0.2	3.5	3.6	0.7	5.3	15.7
40	1.5	0.2	0.1	1.3	2.7	0.2	2.5	8.5
50	1.1	0.3	0.1	1.0	2.1	0.1	2.2	8.1

2.5 Determination of irradiation condition

2.5.1 Bangladesh

Radiosensitivity study of cv. Sabri (AAB)

Radiation sensitivity and post-irradiation recovery were assessed by measuring the survival and propagation rate 40 days after irradiation (Fig. 2-5-B-1). From the result it was found that LD_{50} for Banana cv. Sabri laid in between the gamma irradiation dose of 30 and 40 Gy. So, 35 Gy have been selected as an optimum dose to be used in the experiments for induction of *Fusarium* resistant mutants and getting highest percentage of variants in cv. Sabri.

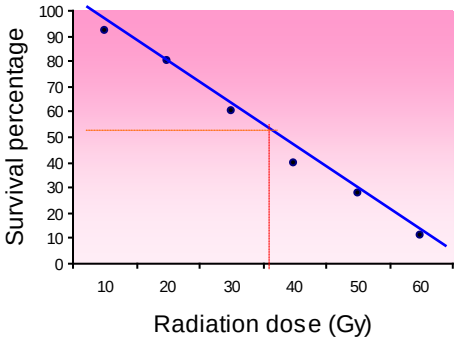


Fig. 2-5-B-1 Radiosensitivity test curve of shoot tips from Banana cv. Sabri

2.5.2 Malaysia

Radiosensitivity test (dose response) for cultivar Berangan was carried out by irradiating meristem tips with a series of gamma ray doses of 0,10, 20, 30, 40, 50, 60, 70, 80, 90, 100Gy. For each dose, 100 meristem explants were irradiated and divided into 5 replicates for statistical analysis. Data for radiosensitivity test was determined by 3 parameters, such as:

1. Plant height (shoot height)
2. % survival of irradiated explants
3. Multiplication rate

Based on % survival of irradiated explants, LD_{50} and LD_{100} were obtained for Berangan:

LD_{50} = 50Gy

LD_{100} = 80Gy

In a mutation breeding experiment, LD_{50} gives an indication value for the response of different types of explants of the same species to radiation so that, a researcher can select the right dose (s) for the main field experiment. Usually, selecting the most effective dose (s) for the main experiment, it is advisable to select a few doses (more than one) which are 20 - 30% lower than the LD_{50} value. Since induction of mutations by radiation for desired mutants is by chance, therefore it is safer to choose more than one dose and the doses that can cause less damage and give higher multiplication rate.

Therefore, based on the percentage survival of irradiated meristems which are 20 - 30% lower than LD_{50}, selected effective doses for the main experiment were 20, 30, 40Gy. Explants from meristem tissues of Berangan were irradiated with gamma rays at 20, 30 and 40 Gy. Irradiated explants were subcultured until M_1V_5 at 4 weeks interval to eliminate chimerism. Irradiated plantlets at different subculture stages were maintained on MS multiplication medium containing 5 mg/l BAP for further regeneration and were incubated in the incubation room at 24 ºC with 16 hours photoperiod. Rooting of the irradiated plantlets was carried out on MS rooting medium containing 2 mg/l IAA. Rooted

plantlets were transplanted to soil-mix in polybags and then transferred to the nursery for hardening process and screened for Fusarium wilt disease using artificial inoculation.

2.5.3 Philippines

The radio-sensitivity response of banana shoot explants was determined by irradiating shoot explants at 0, 5, 10, 20, 25, 30, 40, 60, 80 and 100 Gy. After irradiation, shoots were immediately transferred to shoot multiplication medium (SMM) and multiplied for five subculture cycles (M_1V_1 to M_1V_5). The LD_{50} was determined by the 50% reduction in shoot proliferation rate expressed as % reduction based on the control or unirradiated treatment (Fig 2-5-P-1).

Proliferating shoot cultures were subjected to bulk irradiation the using LD_{50} 20 and 25 Gy. Irradiated shoots were multiplied for five cycles (M_1V_5), rooted, potted out and established in the nursery. Regenerated shoots were rooted, potted out and established in the greenhouse.

Shoot cultures irradiated at low dosage (5 Gy) showed higher multiplication rate while cultures irradiated at higher dosage (40 - 60 Gy) showed more than 80% reduction in the multiplication rate. For cv. Lakatan, the LD_{50} was established at 20 - 25 Gy. For mutation induction, bulk irradiation of shoot tips was done using the LD_{50}. All plants regenerated from irradiation treatments were evaluated for BBTV resistance.

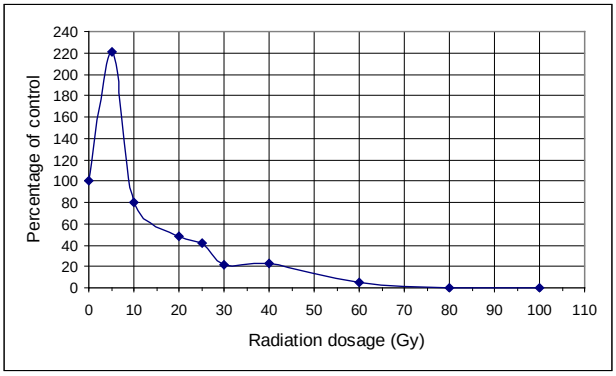


Fig. 2-5-P-1. Effect of increasing dosage of gamma radiation on the multiplication rate of Lakatan (AA) shoot cultures after five subculture cycles. Data are expressed as percentage of control (non irradiated shoot cultures)

2.6 Preparation of pathogens for artificial inoculation

2.6.1 Malaysia

I. *Fusarium oxysporum* (Foc race 4) cultures.

The cultures (4 petri dishes) were kindly provided by Prof. Vikineswary from University of Malaya (Fig. 2-6-M-1). These cultures were then subcultured onto fresh PDA medium supplemented with 1% yeast extract for further multiplication. Subculturing was done by taking one agar slab (0.5 x 0.5cm) containing the fungal mycelium and transferred onto PDA medium (Fig. 2-6-M-2). The cultures were incubated under light at 23 - 25°C in growth chamber. The growth of Foc mycelium will take about 4 - 5 days (Fig. 2-6-M-3).

Fig. 2-6-M-1

Fig. 2-6-M-2

Fig. 2-6-M-3

II. Preparation of *Fusarium* suspension

With a sterile bacterial loop, the surface of the *Fusarium* culture (1 petri dish) was scraped (Fig. 2-6-M-4) and transferred into a sterile test tube containing 10 ml sterile distilled water (Fig. 2-6-M-5). The suspensions were vortex for a few seconds to disperse the mycelium (Fig. 2-6-M-6). Then, another 10 ml of sterile distilled water was added into the suspension and vortex for another few seconds. One petri dish of Fusarium culture = 20 ml solution = 20 plants.

Fig. 2-6-M-4

Fig. 2-6-M-5

Figure 2-6-M-6

III. Observation of *Fusarium* mycelium

A drop of the Foc solution was placed onto a glass slide and stained with Evans Blue (Fig. 2-6-M-7). Observation was done under the light microscope (Fig. 2-6-M-8). Foc mycelium observed under light microscope at 40X magnification (Fig. 2-6-M-9).

Fig. 2-6-M-7

Fig. 2-6-M-8

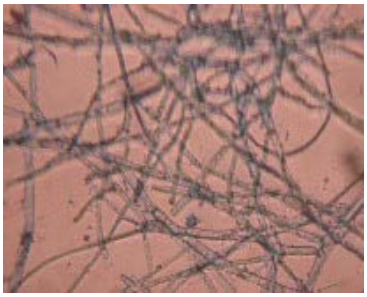


Fig. 2-6-M-9

2.6.2 Philippines

I. Greenhouse Screening for BBTV resistance

Irradiated plants materials regenerated from radio-sensitivity study (5 - 60 Gy) and bulk irradiation (20/25 Gy) were screened for BBTV resistance in the greenhouse using aphid inoculation of the virus. Plantlets about three months from potting and with 4 - 5 fully expanded leaves were subjected to artificial inoculation of the virus using the aphid vector, *Pentalonia nigronervosa.*

The procedure for greenhouse screening consisted of the following steps (Fig. 2-6-P-1):

1. Aphids rearing in healthy *Caladium* or *Diffenbachia* plants
2. Aphids starvation for 30 min
3. Acquisition feeding on infected BBTV infected banana plants for 24h
4. Aphid inoculation to banana plants (24h; 10 aphids/plant)
5. Observation for BBTV symptoms 1 - 9 months after inoculation
6. BBTV indexing by symptomatology, ELISA and PCR
7. Planting the seedling resistant plants without BBTV infection in the field for further evaluation for BBTV resistance and agronomic traits

Plants were kept in the greenhouse and observed for BBTV symptom expression six to nine months after inoculation. The presence or absence of BBTV infection was assessed using symptomatology, ELISA and PCR based techniques. Mutant plants without BBTV symptoms were selected nine months after virus inoculation.

Selected resistant mutant plant were selected, micropropagated and evaluated in the field under high disease pressure or farmer's field until the resistant mutant lines are stable (3 generations of selections)

II. Nematode screening (greenhouse experiment)

Tissue cultured plants from selected G1 mutant lines and check cultivars were evaluated for resistance to nematode *R. similis.* Tissue cultured plantlets were planted in sterilized coir dust: garden soil (1:1 v/v) potting mix. One month after planting, the seedlings (10/line) were inoculated with *R. similis* (1,000 larvae/plant). Evaluation for root damage/dead roots, root health assessment, root necrosis and nematode reproduction (number of juveniles) was done two months after

inoculation. The % dead root was calculated from the number of dead roots divided by the total number of roots multiplied by 100. Root health assessment refers to the assessment of secondary and tertiary roots: 1-0 to 5% of roots healthy, 2-6 to 50% of roots healthy, 3-51 to 95% of roots dead, 4- 96 to 100% of roots dead.

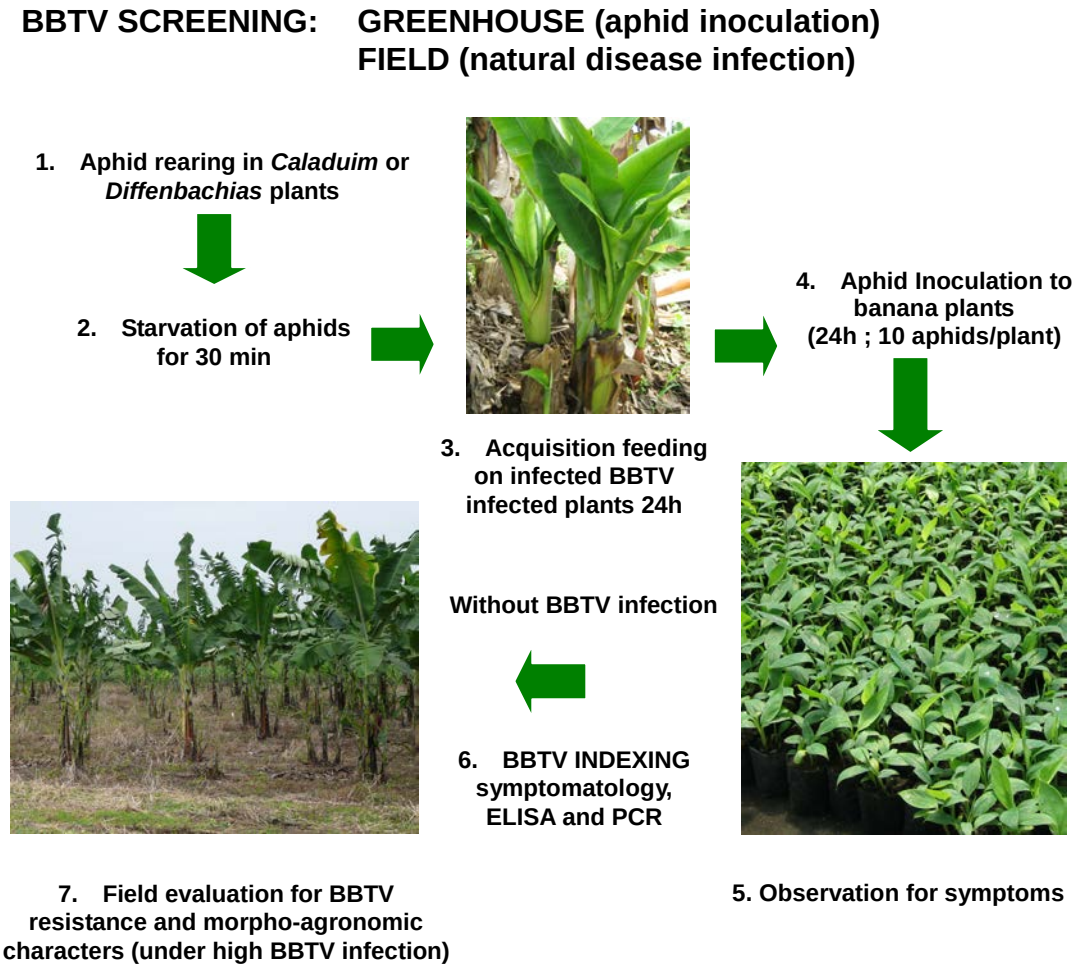


Fig. 2-6-P-1 BBTV disease screening procedure using artificial inoculation of the virus using aphid vector *Pentalonia nigronervosa* (greenhouse) followed by field evaluation under natural disease infection.

2.6.3 Vietnam

I. Effect of artificial inoculation methods on *Fusarium oxysporum* f.sp *cubence* of banana

We used fourth methods with density 10^5 spore/ml. The results showed in Table 2-6-V-1.

Table 2-6-V-1. Effect of artificial inoculation methods on banana plantlets

Method	Wilt rate after 14 days (%)			
	Numbers of repeat			
	I	II	III	Average
wounded	56.6	66.6	60.0	61.0
inf. injection	40.0	43.3	46.6	43.3
root immerge	53.3	46.6	56.6	52.1
cultured hydroponic	76.6	83.3	73.3	77.7

The experiment used four methods to carry out the Foc artificial inoculation on plants but cultured hydroponic is the best method (Fig. 2-6-V-1.a).

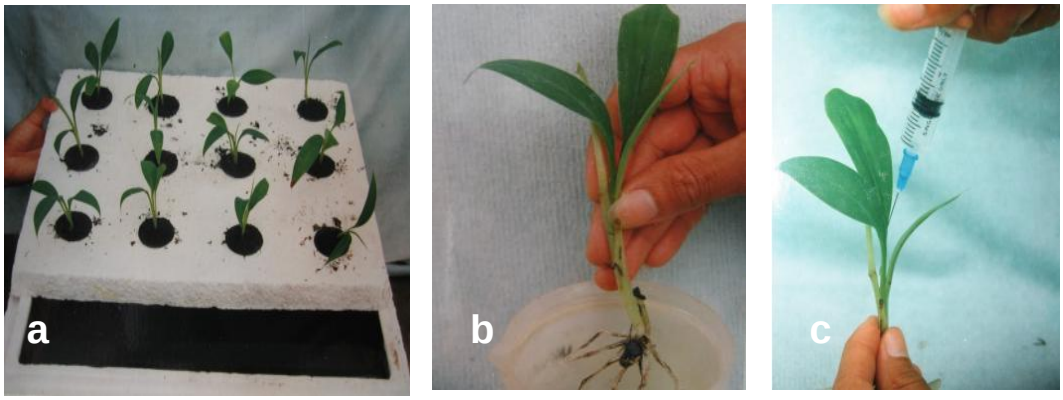


Fig. 2-6-V-1. a. cultured hydroponic, b. root immerge, c. inf. injection

II. Select banana lines anti-Foc by artificial disease inoculation on small banana

The results of selection somatogenic variation lines anti-Foc (*Fusarium oxysporum* f.sp *cubense*)

After 5, 6, 7, 8, 9, 10 times subcultured, shoots were transferred on rooted medium and plants root reached 2 - 3 cm in length and transplanted on the pots in green house. Each formula, we selected 500 plantlets at random. The results showed in Table 2-6-V-2.

Table 2-6-V-2. Synesis rate of bananas in green house

Experiments	Targets	
	No of infected bananas	Synesis rate (%)
Control	285	57.0
I	240	48.0
II	301	60.2
III	189	37.8
IV	241	48.2
V	235	47.0
VI	201	40.2

Selected lines resistant to Foc after gamma treatment (^{60}Co)

The banana plantlets were transferred on pots in green house and were carried out artificial pathogen. We selected 100 plants and observed after 1 month, take immege of infected individual. After continual 3 times, we calculated plants that weren't infected, the results showed in Table 2-6-V-3.

Table 2-6-V-3. Survival rate after artificial inoculation

Dose (Gy)	Number of diseased inoculation		
	The first	The second	The third
0	48/100	22/100	11/100
10	**41/100**	**27/100**	**15/100**
20	**35/100**	**18/100**	**10/100**
30	45/100	29/100	12/100
40	41/100	27/100	14/100

The experiment showed good results in all formulas and infection rate decreased with times of taking artificial inoculation.

Selection of lines enhance resistant to Foc pathogen in green house

The results showed in Table 2-6-V-4.

Table 2-6-V-4. Selection of lines resistant to Foc (green house exp)

Order	Plant lines	No of infected plants	Synesis rate (%)	Levels
1	Control	12	60	MS*
2	I-3	11	55	MS*
3	I-22	11	55	MS*
4	I-35	13	65	MS*
5	I-37	9	45	MR
6	I-41	11	55	MS*
7	I-42	13	65	MS*
8	I-48	15	75	MS*
9	I-53	17	85	S
10	I-57	9	45	MR
11	I-62	15	75	MS*
12	I-68	15	75	MS*
13	I-75	13	65	MS*
14	I-83	13	65	MS*
15	I-84	11	55	MS*
16	I-96	11	55	MS*
17	I-9	14	70	MS*
18	I-25	10	80	S
19	I-36	4	20	R
20	I-37	2	10	R
21	I-45	11	55	MS*
22	I-47	3	15	R
23	I-58	4	20	R
24	I-73	4	20	R
25	I-87	9	45	MR
26	I-92	11	55	MS*
27	I-7	13	65	MS*
28	I-25	14	70	MS*
29	I-35	6	30	MR
30	I-47	2	10	R
31	I-51	2	10	R
32	I-62	7	35	MR
33	I-68	3	15	R

34	I-73	5	25	R
35	I-77	3	15	R
36	I-82	5	25	R
37	I-88	7	35	MR
38	I-95	1	5	R
39	I-6	13	65	MS*
40	I-24	9	45	MR
41	I-34	11	55	MS*
42	I-38	16	80	S
43	I-46	8	40	MR
44	I-53	4	20	R
45	I-58	3	15	R
46	I-67	4	20	R
47	I-75	6	30	MR
48	I-78	8	40	MR
49	I-79	7	35	MR
50	I-85	6	30	MR
51	I-93	4	20	R
52	I-94	4	20	R

S: sensitive; **MS:** moderate sensitive; **MR:** moderate resistance; **R:** resistance

The banana plants weren't infected by *Fusarium oxysporum* f.sp. *cubense*, that were transferred on *in vitro* medium and propagated

2.7 Inoculation of pathogens and observation of symptom development in the laboratory and/or nursery

2.7.1 Malaysia

Four effective methods for screening of Fusarium wilt disease in banana had been established using artificial inoculation with *Fusarium oxysporum* f. *cubense* (Foc Race 4). These methods can be applied for pre-screening of irradiated *in vitro* plantlets or seedlings to select for resistant/tolerant lines before they can be transferred to the field for final selection of potential mutant lines.

I. Methods of screening:

1. *In vitro* screening - dipping of roots of *in vitro* plantlets in *Fusarium* spore suspension
2. Double-tray technique - inoculation of tissue culture plantlets with *Fusarium* spore suspension in the nursery
3. Planting of 2 months old hardened plants in soil medium inoculated with *Fusarium* mycelium
4. Field screening - planting of treated plants in *Fusarium* 'hot spot' area.

II. Establishment of screening methods for Fusarium wilt disease:

1. Dipping method:

This is done by soaking of rooted *in vitro* plantlets (4 - 5 inches tall) in *Fusarium oxysporum* suspension (10^6 spores/ml) for 1 - 2 hrs and later transferred to sterile sand media in the greenhouse. Inoculated plants were observed for disease symptoms such as yellowing of the leaves and for confirmation, the lower part of the plant is cut across which shows purple discoloration of the pseudostem.

Rooted *in vitro* plantlets as explants for dipping method

2. Double tray method:

This technique consists of 2 separate plastic tray or container: First tray contains sterile sand media whereby rooted plantlets were hardened for 3 - 4 weeks. After the plants were hardened, *Fusarium oxysporum* spore suspension (10^6 spores/ml) is poured into the tray containing plantlets. The treated plants were watered daily and excess water containing the spores is collected in the second tray placed below the first tray. This technique is most suitable since excess spores are contained and will not escape to the drain or ground which might contaminate the area. Similar symptom for yellowing of the leaves will be recorded and discoloration of the pseudostem gives confirmation for infected plant.

Double tray method

3. Nursery screening method:

Rooted *in vitro* plantlets were hardened in the greenhouse using individual polybags containing sterile sand media for 8 weeks. Later the hardened plants were transferred to fibre-glass trough

(1x2m) containing mixture of coir dust which had been inoculated with *Fusarium oxysporum* spore suspension (10^6 spores/ml) for 2 weeks. Evaluation for disease infection is done 4 - 6 weeks after planting. Similar symptom for yellowing of the leaves will be recorded and discoloration of the pseudostem gives confirmation for infected plant.

Nursery screening method

Tolerant/Resistant plants

Susceptible plants

4. Field screening:

Those inoculated plants that survived from the above screening methods are transferred to hot spot. Resistant plants are multiplied and transferred back to hot spot for at least 2 - 3 generations. Similar symptom for yellowing of the leaves will be recorded and discoloration of the pseudostem gives confirmation for infected plant.

Field screening of irradiated seedlings and leaf-yellowing, typical symptom for Fusarium wilt disease starts to develop after 4 months.

2.7.2 Philippines

I. Greenhouse screening BBTV resistance

A total of 6,012 plants regenerated from gamma irradiation treatments (radio-sensitivity study using 5 - 60 Gy and bulk irradiation at 20/25 Gy) were evaluated for resistance to BBTV using artificial inoculation of the virus by aphid transmission. Of the 6,012 irradiated plants screened in the greenhouse, 114 plants were selected without symptoms of BBTV nine months after virus inoculation *(Table 2-7-P-1)

Table 2-7-P-1. Total number irradiated plants screened in the greenhouse for BBTV resistance using aphid inoculation of the virus

Radiation Treatment	Total no. of plants screened for BBTV resistance.	No. of plants without BBTV symptoms	
		6 mos. after inoculation	9 mos. after inoculation
Radio Sensitivity (5-60 GY)	1,847	78	72
TC Control	161	0	0
Bulk irradiation (20/25)	4,165	79	42
TC control	388	0	0
TOTAL Irradiated plants	**6,012**	**157**	**114**
TOTAL TC Control	**549**	**0**	**0**
BBTV indexing ELISA PCR	- (negative) in 33/45 plants sampled (73%) - (negative) in 18/25 plants sampled (72%)		

II. Evaluation for nematode resistance under greenhouse condition

Based on the results on root damage, almost all roots of mutant lines 2-45, 4-45, 10-45 were healthier than the TC control (non-irradiated) (Table 2-7-P-2). In case of mutant line 5-45, although the % of dead roots was 0, most of the secondary and tertiary roots were dead (3.0). For % root necrosis, mutant line 4-45 had the highest value (90%) followed by the TC control Lakatan (73.6%); The values obtained by these two entries are even higher than the susceptible check Grand Naine. For number of juveniles, mutant line 4-45 had the lowest (20) followed by PR 2-45 (298).

Based on the results, mutant lines 2-45, 4-45, and 10-45 were resistant to *R. similis* under greenhouse condition. Due to lack of funds, field evaluation for nematode resistance was not conducted.

Table 2-7-P-2. Root damage assessment and nematode reproduction of irradiated and unirradiated banana cv Lakatan inoculated with *Radopholus similis*

Mutant line	% Dead Roots*	Root Health Assessment**	%Root necrosis	***Number of Juveniles
2-45	7.1	2.2	14.2	298
4-45	0	2.0	6.5	20
5-45	0	3.0	90.0	7,269
10-45	0	2.0	8.0	2,020
Unirradiated Lakatan (TC control)	41.5	3.0	73.6	2,317
Yangambi Km 5 (R check)	**0**	**1.0**	**15.0**	**58**
Grand Naine (S check)	**28.3**	**2.8**	**60.0**	**2,959**

*%Dead roots= number of dead roots divided by total number of roots multiplied by 100.
**Root Health Assessment refers to the assessment of the secondary and tertiary root: 1.0-5% of roots healthy, 2-6 to 50% of roots healthy, 3- 51 to 95% of roots dead, 4- 96 to 100% of roots dead.
+++ Number of juveniles taken from one gram of root samples.

2.8 Inoculation of pathogens and observation of symptom development in hot spots

2.8.1 Malaysia

I. Field screening of irradiated plants in farmer's field

Rooted *in vitro* plantlets were first treated with *Fusarium oxysporum*, Foc race 4 (10^6 spores/ml) suspension cultures using double-tray method. Plants that survived and which showed no symptoms of infection (yellowing of the leaves and discoloration of pseudostems) were selected for further screening in the field. A total of 1,115 treated plants (mutant lines) from doses of 20, 30 and 40Gy including 183 control plants were planted in farmer's field in May 2007 to screen for Fusarium wilt. Numbers of seedlings used for the field screening are;

1. Control: 183 seedlings
2. 20 Gy: 458 seedlings
3. 30 Gy: 343 seedlings
4. 40 Gy: 314 seedlings

II. Screening for virus using ELISA Test

Both tissue-cultured treated plants and control were screened for the presence of the virus. About 10% of the total population of the treated and control tissue culture plants were sent to Crop Protection & Plant Quarantine Services Division, Department of Agriculture, Malaysia for laboratory test against the following viruses:

1. *Banana bunchy top virus* (BBTV)
2. Banana streak virus (BSV)
3. *Cucumber mosaic virus* (CMV)
4. *Banana bract mosaic virus* (BBrMV)

III. Scoring for disease symptoms and evaluation of agronomic characters

Disease scoring for Fusarium wilt was done after 4-months planting period whereby each individual treated plants and control plants were analyzed for symptoms of yellowing of the leaves, discoloration of pseudostems and other factors that affects growth of the plants. For the control plants, out of 183 plants tested, 66 plants (36%) showed symptoms of *Fusarium* infection. On the other hand, for treated plants, the following observations were made:

IV. Disease scoring: susceptibility to Fusarium wilt

1. Control: 66 plants (36%)
2. 20 Gy : 10 plants (4%)
3. 30 Gy : None (0%)
4. 40 Gy : None (0%)

The percentages of Fusarium wilt infection in treated plants were observed to be very low. This might be because of the resistance or this could probably due to many of the plants might have escape

infection due to environmental factors. Therefore, it is suggested that second or even third field screening should be carried out in order to select for mutant plants which are stable and resistance to Fusarium wilt disease. Besides screening for Fusarium wilt, desired agronomic characters such as early flowering and short stature plant were selected as below:

V. Selection for agronomic characters:

1. 20 Gy: 458 seedlings
   Early fruiting: 52 plants (11%)
   Dwarf: 5 plants (1%)
2. 30 Gy: 343 seedlings
   Early fruiting: 19 plants (5%)
   Dwarf: 21 plants (6%)
3. 40 Gy: 314 seedlings
   Early fruiting: None (0%)
   Dwarf: 75 (24%)

Three potential mutants tolerance to Fusarium wilt disease having improved agronomic characters such as high yield, early flowering and short stature were selected from a total population of 1,115 treated plants as follows:

20 Gy: 2 mutants of high yield and early flowering
40 Gy: 1 mutant of high yield and short stature.

VI. Stability test for selected mutants

At present, a total of 1,000 plants from 20 and 40 Gy populations which had been micropropagated, were further field tested in the second location in farmer's plot for stability against Fusarium wilt disease.

2.8.2 Philippines

I. First field evaluation BBTV resistance under high disease pressure

The selected seedling resistant M_1 mutant plants (114) were planted in the field under high BBTV infection. After 21 months of exposure to natural BBTV infection, 64 irradiated M_1 mutant plants were selected (Table 2-8-P-1; Fig. 2-8-P-1 and 2). Suckers of selected mutants were collected and micropropagated for further field evaluation.

Table 2-8-P-1. Total number of M_1 BBTV resistant mutant plants selected after 30 months of evaluation (greenhouse artificial inoculation of the virus followed by and natural field disease infection).

Radiation treatment	Total no. of irradiated plants evaluated	No. of selected M_1 mutant BBTV resistant plants (M_1 plants without BBTV symptoms)	
		After greenhouse evaluation	After field evaluation
Radio sensitivity experiment (5 - 60 Gy)	1,847	72	38
Bulk irradiation 20/25 Gy	4,165	42	26
TOTAL irradiated plants	**6,012**	**114**	**64**
% M_1 resistant plants			**1.06%**

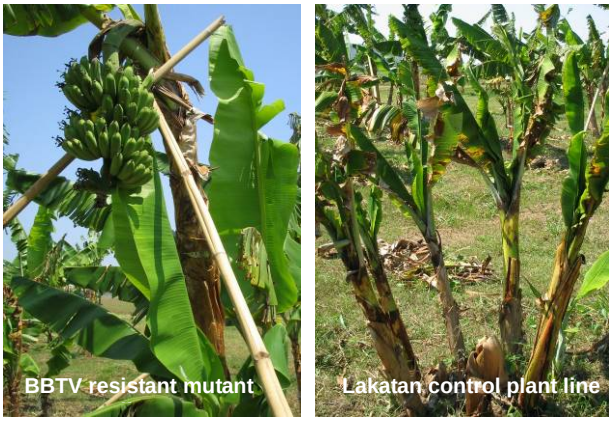


Fig. 2-8-P-1. BBTV resistant mutant line and Lakatan control plant after field evaluation

Fig. 2-8-P-2 Remaining Generation 1 suckers of selected resistant plants without BBTV symptoms at harvest. Cut plants were all BBTV infected.

II. Second field evaluation (generation 1 and 2 plants) for BBTV resistance

Thirty-two selected mutant lines (out of 64 mutant lines from initial selections) were planted in experimental field under high BBTV infection. Plants were planted in a randomized complete block design with two blocks and three replications with five plants per replication or a total of 30 plants per mutant line. Each block was surrounded with BBTV infected plants as sources of inoculum. All infected experimental plants were also kept in the field to provide additional sources of inoculum. The incidences of BBTV infection were taken every three months until harvest. Agronomic data were taken at harvest.

Out of 32 mutant lines evaluated 10 mutant lines with % BBTV infection less than control plants 17 months after field planting were selected (Table 2-8-P-2).

Suckers from the 33 line/mat selections from the 10 mutant lines were collected indexed and micropropagated and further evaluated for stability of resistance of generation 3 plants.

Table 2-8-P-2. The % BBTV disease incidence in selected BBTV resistant mutant lines 3 to 17 MAP.

Mutant line	% BBTV incidence (G1 and G2 plants)* month after planting (MAP)					% BBTV-free fruiting (harvested) plants* month after planting (MAP)		
	3	7	11	15	17	12	14	17
25-28	0	0	6.9	17.2	31.0	6.7	53.3	96.0
23-30	0	0	3.3	13.3	33.3	3.3	43.3	70.0
2-45	0	3.4	14.3	21.4	50.0	0.0	26.7	46.7
13-30	0	0	3.8	7.7	50.0	3.3	46.7	66.7
22-28	0	6.7	6.7	16.7	50.0	0.0	60.0	76.7
23-28	3.4	14.3	21.4	46.4	57.1	10.0	40.0	76.7
7-29	6.9	7.1	21.4	28.6	57.1	6.7	46.7	70.0
23-45	3.6	3.6	3.7	14.8	66.7	0.0	56.7	80.0
9-28	6.7	18.5	37	59.2	66.7	43.3	50.0	63.3
4-28	0	3.7	3.7	26.9	69.2	3.0	50.0	76.7
TC Control	1.4	14.1	18.3	29.6	71.8	0.0	14.1	32.4

*Combined G1 and G2 plants

III. Selection of Promising Generation 3 BBTV Resistant Mutant Lines for Multi-location Field Evaluation and Characterization of Disease Resistance Mechanism

10 lines were selected based on BBTD incidence in the field, % BBTV-free fruiting plants and the results obtained from the studies on mechanism of resistance. The ten selected resistant lines namely lines 13-30-2, 7-29-1, 22-28-2, 23-28-7, 6-30-2, 9-28-2, 9-28-3, 9-29-1, 23-30-2, and 28-30-2 showed low disease incidence in the field, and/or obtained low disease incidence amid the presence of insect vector compared to LKD control plants. The selected lines were composed of nine mutant lines and one somaclonal variant (6-30-2). Past evaluations regarding stability of resistance in succeeding generations were also considered in the process of selection.

IV. Studies on the mechanism of resistance in selected G3 mutant lines.

A. Aphid preference (Insect vector-host relationship)

The aphid preference on 10 selected mutant lines was determined using the free choice or no choice feeding of the aphid. Five lines were found significantly less preferred by the aphids namely: 6-30-2. 9-28-2, 9-28-3, 23-28-7 and 28-30-2 (Table 2-8-P-3). Two lines (9-28-2 and 9-28-3) were consistently less preferred by the aphids in both free choice and no choice feeding.

Table 2-8-P-3. Relationship between aphid preference (aphid colony count) and BBTV incidence in selected mutant lines

Mutant lines	Aphid colony count at the peak of colonization (3^{rd} week of feeding)	
	Free choice feeding of the vector	No choice feeding of the vector1
28-30- 2	40**	62*
13-30 -2	22	65*
9-28 3	12*	36*
22-28 -2	NT	65*
9-28-2	12*	49*
6-30 -2	27	59*
7-29-1	14*	110
9-29 -1	1*	83
23-28-7	NT	16*
23-30-2	40**	76*
LKD control	23	127

*Significantly different at P= 0.05%

** Significantly different at P=0.05%

NT- not tested due to lack of planting materials

B. Reaction of mutant lines to artificial inoculation of the virus

The reaction of 10 selected mutant lines to BBTV was determined through artificial (aphid) inoculation of the virus. Five lines consistently showed low BBTD incidence (<50%) in repeated artificial inoculation trials namely: 23-30-2, 28-30-2, 23-28-7, 13-30-2 and 9-28-2 (Table 2-7-P-4). Expression of the virus ranged from bunchy top growth of the shoot, chlorosis of the youngest fully expanded leaf and streaks on petiole and pseudostem (Fig. 2-8-P-3).

Seven lines showed the % BBTD incidence lower than the LKD control plants. Line 28-30-2 showed the lowest BBTD incidence (19.6%) followed by lines 13-30-2 (25.0%), 9-29-1 (32.8%) and 22-28-2 (38.4%). Likewise, lines 28-30-2 and 13-30-1 had the longest incubation period of up to 6 weeks. Other lines including the LKD control had 4 - 5 weeks incubation period. The symptoms observed on infected plants ranged from bunchy top, rosette growth, and marginal chlorosis.

Based on ELISA (OD_{405nm}) reading (Table 2-8-P-6), all mutant lines except 9-29-1 had min OD reading less than that of the LKD control plants. On the other hand, for max OD reading only lines 13-30-2 and 7-29-1 had max OD reading less than that of the control plants. The results showed that some mutant lines had low virus titer, suggesting some degree of resistance to virus.

Table 2-8-P-4. Reaction of the ten lines to artificial inoculation of BBTV under greenhouse condition

Mat/Line selection	% BBTD incidence	Incubation period (WAI)	Symptoms	ELISA (OD405nm) Range	
				Min	Max
28-30- 2	19.6	4-6	bunchy top,	0.189	1.276
13-30-2	25.0	5-6	rosette	0.1735	0.956
9-28 3	50.0	4-5	bunchy top, marginal chlorosis	0.1885	1.605
22-28 -2	38.4	5	rosette	0.1875	1.57
9-28-2	50.0	4-5	bunchy top	0.1785	1.267
6-30 -2	55.6	4-5	bunchy top	0.1835	2.2915
7-29-1	57.5	4-5	bunchy top, rosette	0.1815	1.128
9-29-1	32.8	4-5	bunchy top, rosette	0.203	1.545
23-28-7	45.0	4-5	bunchy top	0.1705	1.336
23-30-2	70.7	4-5	bunchy top, rosette	0.1785	1.68
LKD control	58.3	4-5	bunchy top	0.194	1.287

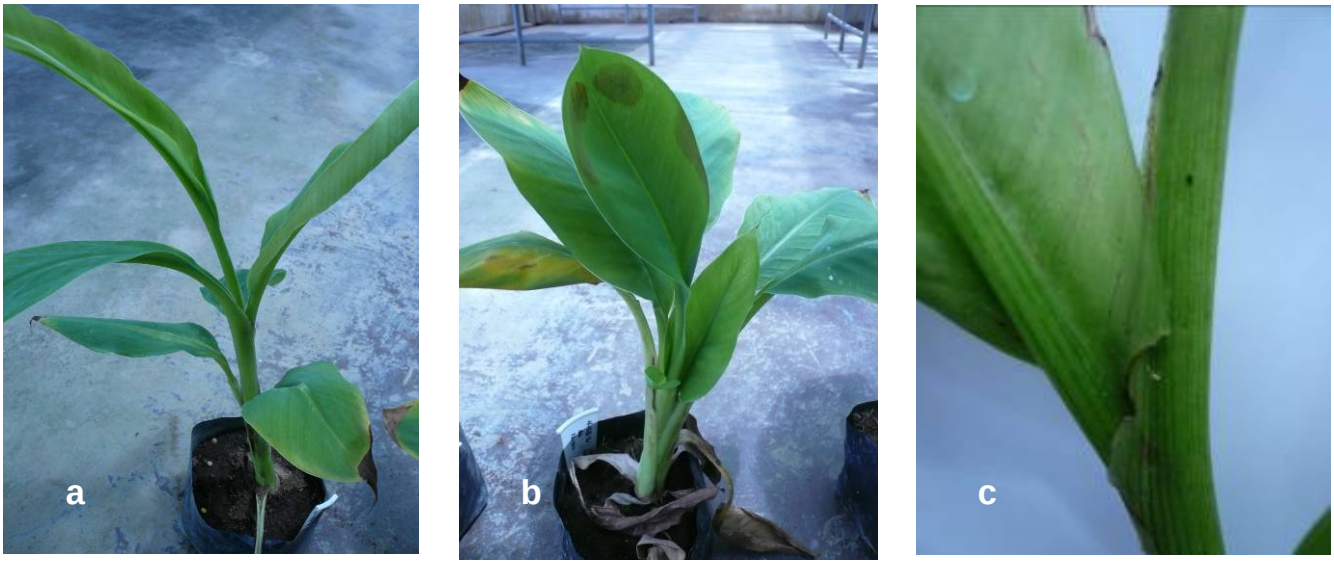


Fig. 2-8-P-3 Symptoms of BBTD observed under greenhouse condition:
a. Chlorosis on the youngest full expanded leaf,
b. Bunchy top growth of the shoot,
c. Clear streaks on the leaf petiole and in the pseudostem

V. Multi-location field evaluation of selected mutant lines

Due to limited funds, only the top five promising mutant lines namely 9-28-2,9-28-3,13-30-2, 22-28-2and 28-30-2 were planted in multi-location trials.

Low incidences of the banana bunchy top disease were observed in all trial sites despite the presence of infected plants and vectors in the vicinity of trial sites. The highest percentage incidence of the disease was recorded at 11.6% (in the case of the Bay, Laguna trial site). In Cavite, which is considered a hot spot for BBTV infection (Table 2-8-P-5 and 6), the diseases did not spread intensively within the trials sites and the infection occurred at random. From planting until 19 months, there was very little spread of the disease with time within the experimental area. The aphid vectors within the experimental site were observed in lines 13-30-2, 22-28-2 and 28-30-2.

The low incidence of BBTD in trial site could be due higher number of resistant plants planted in the area; approx. 70% of the total plants showed varying degrees of resistance. Furthermore, the random arrangements of test plants with different resistance mechanisms in the field, in some way prevented the rapid spread of the vector and virus within the experimental site. Regular rouging of infected plants within the trial sites prevented secondary infection. Further studies on the use and deployment of resistant lines as part of the BBTD management strategies are needed.

Table 2-8-P-5. Percentage incidence of BBTD in five mutant lines of banana cv. Lakatan selected for multi-location trial planted in Indang, Cavite (CAV 1).

Mutant line	Mat/Line selection	No. of plants	% Disease incidence (MAP)									BBTV- free fruiting plants (%)
			6	8	10	11	12	14	16	18	19	
9.28	9.28.2	60	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	38
9.28	9.28.3	60	0	0	0	0	1.6	1.6	1.6	1.6	1.6	23
13.30	13.30.2	60	1.6	1.6	1.6	1.6	3.3	3.3	3.3	3.3	3.3	35
22.28	22.28.2	60	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	3.3	26
28.30	28.30.2	60	1.6	1.6	1.6	1.6	3.3	3.3	3.3	3.3	3.3	35
Control	LK	60	0	0	0	0	0	0	0	0	0	28
Border	LK	110	1.8	2.7	2.7	2.7	2.7	2.7	2.7	2.7	2.7	20
Selected Resistant Lines		300									2.7	
LK (Control + Border)		170									1.8	
Whole experimental area		470									2.3	

Table 2-8-P-6. Percentage incidence of BBTD in five mutant lines of banana cv. Lakatan selected for multi-location trial planted in Indang, Cavite (CAV 2).

Mutant line	Mat/Line selection	No. of plants	% Disease incidence (MAP)									BBTV free fruiting plants* (%)
			6	8	10	11	12	14	16	18	19	
9.28	9.28.2	60	0	0	1.6	1.6	3.3	3.3	3.3	3.3	3.3	67
9.28	9.28.3	60	1.6	3.3	3.3	3.3	5	5	5	5	5	42
13.30	13.30.2	60	3.3	5	5	5	5	5	5	5	5	47
22.28	22.28.2	60	0	0	0	0	0	0	0	0	0	53
28.30	28.30.2	60	0	3.3	3.3	3.3	3.3	5	5	5	5	58
Control	LKD	60	0	0	1.6	1.6	1.6	3.3	3.3	3.3	3.3	28
Border	LKD	80	1.25	1.25	2.5	2.5	2.5	2.5	2.5	2.5	2.5	30
Selected Resistant Lines		300									3.7	53
LKD (Control + Border)		140									2.9	
Whole experimental area		440									3.4	

*Observations were made on mother plants

VI. Agronomic and Yield Performance of Five Selected Lines in Multi-location Trial

The BBTV resistant mutant lines were comparable with LK control in terms of plant height, girth, total number of fruits per bunch, number of hands per bunch and number of fingers per hand (Table 13;). Number of days to flowering was significantly early for lines 13-30-2 (26 says earlier), 22-28-2 (21 days earlier) and 28-30-2 (17 days earlier). Mutant lines had a mean of 6 - 7 hands/ bunch and 17-18 fingers/hand. The crop stand in farmers field is shown in **Fig. 2-8-P-6**.

Table 2-8-P-7. Agronomic and yield parameters taken from BBTV resistant mutant lines (mother plants, mean from multi-location trials)

Mutant line	Mat/Line selection	Plant height* (cm)	Girth (cm)**	No. of days to flowering	No. of fruits per bunch	No. hands/ bunch	No. of fingers per hand
9.28	9.28.2	342.6	56.1	408	123	6.6.	17
9.28	9.28.3	340.8	54.8	408	128	6.5	17
13.30	13.30.2	334.5	54.1	397*	119	6.3	17
22.28	22.28.2	347.0	55.5	402*	130	6.6	18
28.30	28.30.2	342.3	55.3	406*	130	6.5	18
LK control	LK	319.6	52.3	423	108	6.0	19
t- test P=0.05		NS	NS	SD	NS	NS	NS

*Measurements taken at flowering

** Measurements taken 1m from the base of the pseudostem at flowering

Fig. 2-8-P-4 Crop stand and bunch yield of BBTV resistant mutant line