3. General Discussion

3.1 For application of mutant lines in the future

3.1.1 Bangladesh

I. *In vitro* mutagenesis of cv. Sabri

Mutation techniques in conjunction with *in vitro* culture (e.g. irradiation of shoot tips and subsequent regeneration of plants) have been suggested suitable alternative for the improvement of banana including disease resistance (Novak *et al*., 1990). A total of 1,120 irradiated shoot tip explants in seven batches were cultured on regeneration media MS + 5.0 mg l^{-1} BA + 0.2% Ads (Table 3-1-B-1). An average of about 4 M_1V_4 plantlets ((Fig. 2-3-B-1g) were regenerated from each irradiated shoot tip explant after three repeated subculturings in the same medium whereas in case of control (un-irradiated) it was 10-12 plantlets/explant ((Fig. 2-3-B-1h). Repeated vegetative propagation is needed to dissociate chimeras, but the minimum number of cycles required is unknown (Roux, 2004). Altogether 4334 M_1V_4 plantlets were harvested from irradiated shoot tip explants and transferred to the hormone free MS media for root induction (Fig. 2-3-B-1i). After rooting the plants were transplanted to the poly-bags filled with sterilized soil mix and kept in the hardening room with controlled light, temperature and humidity for 4 - 6 weeks (Fig. 2-3-B-1j &1k). During hardening, about 85% plantlets were survived. After proper acclimatization, out of survived 3,744 numbers of M_1V_4 plantlets, about 2,664 numbers of M_1V_4 plantlets were transferred to the earthen pots containing *Fusarium* infected soil which was collected from the hot spots and kept under greenhouse condition for screening ((Fig. 2-3-B-1l &1m) and found heavily infested (Fig. 3-1-B-1a) and died after 2 to 3 months. The rest of about 1000 plantlets were planted in the hot spot affected field (Fig. 3-1-B-1b) and also found to showed symptoms of *Fusarium* and died (Fig. 3-1-B-1c,d &e) after 6 to 8 months. No *Fusarium* tolerance line was obtained during the study of 4 years.

Table 3-1-B-1.

Response of irradiated and un-irradiated shoot tip explants of cv. Sabri to MS medium supplemented with 5.0 mg/l BA and 0.2% Ads on multiple shoot regeneration after three subcultures in the same medium.

Batch No.	No. of shoot tip explants inoculated		No. of plants regenerated		No. of plants transferred to the Greenhouse/Field for screening	
	Control	Irradiated	Control	Irradiated	Control	Irradiated
1	10	160	105	608	40	505
2	10	160	111	656	40	551
3	10	160	109	576	40	478
4	10	160	115	624	40	537
5	10	160	122	706	40	593
6	10	160	140	660	40	560
7	10	160	90	504	40	520
Total	70	1120	792	4334	280	3744

II. Anther culture of diploid banana

The number of anthers inoculated and response of anthers to callus induction in different culture media is presented in Table 2. A total of 698 anthers were cultured in MS1 medium and five (0.7%) of them responded to produce calli after 7 - 9 weeks of culture in this medium. These calli were creamy and starchy and failed to regenerate any plants when transferred to regeneration media. On the other hand, a total of 2,640 and 2,010 anthers were plated onto MS2 medium and N6 media respectively. Anthers in both the media responded to produce calli. The days required to callus induction ranged from 4 - 8 months. The frequency of callus formation accounted to 11 (0.4%) for MS2 and 5 (0.2%) for N6 media (Table 3-1-B-2). These calli were white, compact (Fig. 2-4-B-2f) and was found to morphogenic response when transferred into fresh MS medium containing Morel vitamins, 0.5 mg l^{-1} BA and 0.4 mg l^{-1} IAA. In some cases, embryoids were produced in this medium. Plant regeneration occurred when these calli/embryoids were sub-cultured on to the same media but with 0.1 mg l^{-1} IAA instead of 0.4 mg l^{-1} IAA (Fig. 2-4-B-2i, j & k). In one instance, regeneration of shoot in callus induction medium was noticed on N6 based medium (Fig. 2-4-B-2g&h). Regenerated shoots were rooted on growth regulator free MS medium. A total of 49 plantlets were regenerated, out of which 32 survived in the potted condition (Fig. 2-4-B-2l) during acclimatization and finally transplanted to the field for further evaluation (Fig. 2-4-B-2m). Cytological study of the root tips was carried out on 6 anther-derived plants. It was very difficult to separate and clearly distinguish each and every chromosome for counting under normal compound microscope due to its very tiny size. But it seemed that all of them possessed diploid number of chromosomes (doubled haploids, 2n =22, Fig. 2-4-B-2n & o). The growth of the doubled haploid plants was very slow and stunted and after 18 months their average height becomes only around 2.0 - 2.5 meter and started flowering after 8 to 12 months and still flowering after 18 months old. But the control diploid plants reached up to 4.5 - 5.5 meter in height and will set fruit within a year.

Table 3-1-B-2. Effects of media composition on number of anthers responded to callus induction and plant regeneration in *Musa balbisiana* (BB) variety Bichikala

Type of callus inducing media	No. of anthers inoculated	No. of anthers responded (%)	No. of green plants regenerated from calli on MS + 0.5 mg l^{-1} BA + 0.1 mg l^{-1} IAA medium
MS (Morel Vit.) + 2.5 mg l^{-1} 2,4-D + 1.0 mg l^{-1} kinetin	698	05 (0.7)	0
MS (Morel Vit.) + 1.0 mg l^{-1} BA + 0.4 mg l^{-1} IAA + 500 mg^{-l} CH	2,640	11 (0.4)	36
N6 (Morel Vit.) + 1.0 mg l^{-1} BA + 0.4 mg l^{-1} IAA + 500 mg^{-l} CH	2,010	05 (0.2)	13

From the results it was observed that anthers containing pollen in the uni-nucleate stage responded to produce calli. In majority of the crops, pollen in the uni-nucleate stage is considered as the optimum stage of development for culture of microspores (Alemano and Guiderdoni, 1994). The production of diploid banana plants could be a consequence of spontaneous chromosome doubling in haploid cells. The other possibility is the regeneration of diploid anther tissues. But in monocots, participation of somatic tissues of anther in organogenesis has been rarely reported (Assani *et al*., 2003). As described in (Assani *et al*., 2003), 41 plants were found haploid out of 147 plantlets obtained from four genotypes of diploid banana (BB) varieties. This reference also described that the frequency of haploid plant production is depended on genotype. In barley, up to 90% of the anther derived plants have been found diploid (Lyne *et al*., 1986). The absence of within family segregation results in doubled haploids being a valuable source of cultivar production (Kasha and Reinbergs, 1975).

3.1.2 Malaysia

Mutations are the ultimate source of genetic variation. They provide the raw materials which other factors of evolution act and therefore all new species ultimately arose from mutation. Mutations were defined as heritable changes in the genetic material not caused by recombination or segregation. In all breeding procedures, major steps to be followed are as follows:

1. Choice of starting materials
2. Choice of mutagen (radiation or chemical)
3. Choice of suitable explants for mutagenesis
4. Radiosensitivity test
5. Selection of effective doses for main experiment
6. Desired traits for improvement
7. Methods of screening
8. Selection of the genotypes required
9. Maintenance and propagation of new cultivars

For banana breeding program, the identification of an efficient screening approach is essential in a mutation breeding approach since a high mutation induction rate, followed by a poor screening approach could result inefficiency and ultimately project failure. In general, selection of a trait, such as resistance to diseases, it is recommended to screen more than 1,000 plants (sometimes more than 10,000 plants) in the field or in a greenhouse to achieve the ultimate objectives. Following selection of potential genotype with desired traits, it is important to verify if the selected trait is heritable. In order to assess the heritability of a newly identified mutation, it will be necessary to conduct multi-location trials for a minimum period of 2-3 generations.

Banana is the second most commonly grown fruit crop in Malaysia. Overall banana production has decreased due to the increasing threat of Fusarium wilt disease, high labour costs and marketing

issues. This program was initiated to improve banana cultivars by induced mutations and biotechnology, especially to produce mutant varieties with improved traits such as tolerance to *Fusarium* wilt disease, high yield, early maturity, and short stature plants. Banana shoot-tip cultures were most suitable for micropropagation for large-scale plant production and most commercial companies have adopted this method for mass propagation of *in vitro* plantlets. However, the cost of production of *in vitro* plants could be reduced by low-cost micropropagation.

To date, somatic embryogenesis had been successfully employed for plant regeneration and could be an alternative method for mutation induction in banana improvement. Since mutation is a single cell event, therefore the use of somatic embryos for mutation induction could lead to the production of higher percentages of mutation and formation of solid mutants. In comparison, the use of meristem cultures had been proven to produce chimerism, whereby irradiated cultures had to be sub-cultured for a few generations to minimize chimerism. Somatic embryos obtained could be used for large scale propagation of plants, target materials for genetic engineering and the production of synthetic seed. Cell suspension could be established from the embryos for mass propagation.

The present research program was initiated with the objective of improving the important dessert bananas in Malaysia, particularly Pisang Berangan. This includes production of resistance or tolerance to Fusarium wilt or Panama disease, short plant stature, early fruiting, and high bunch weight. Banana cultivars are vegetatively propagated clones and are generally triploids and sterile. Tissue culture techniques have been exploited for (a) propagation of selected lines or natural variants; (b) generation of somaclones; (c) production of meristem pieces for *in vitro* mutagenesis and polyploidy induction; and (d) zygotic embryo culture to generate seed progenies for genetic and molecular studies, e) Pre-screening for diseases using artificial inoculation using the pathogens and e) Screening and selection of desired genotypes with improved traits in hot spot, f) Multi-location trials for stability of selected genotypes, g) Molecular characterization of selected mutants using marker-assisted selection, h) Use of micropropagation techniques for multiplication and maintenance of potential mutants, 1) Registration of potential mutants for release and commercialization.

3.1.3 Philippines

Ten promising BBTV resistant mutant 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) were selected after three generations of continuous field evaluation under high BBTV infection.

The reactions of the selected mutant lines to the aphid vector were determined. Mutant lines 13-30-2, 9-28-2, 9-28-3, 6-30-2 and 23-28-28-7 were significantly less preferred by the aphids while lines 7-29-1 and 9-29-1 and Lakatan control plants were equally preferred by the vector. The reactions of selected mutant lines to artificial inoculation of the virus using viruliferous aphids were likewise evaluated. Seven lines consistently showed low BBTV incidence compared with the Lakatan control plants. Line 28-30-2 showed the lowest BBTV 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 Lakatan control had 4-5 weeks incubation period. The symptoms observed on infected plants ranged from bunchy top, rosette growth, and marginal chlorosis.

Results of the vector-virus-host relationship study using the free choice or no choice feeding of the vector showed that BBTV resistance in some mutant lines (28-30-2, 13-30-2, 22-28-2 and 23-28-7) was due to less preference of the vector to the host plant. On the other hand, for lines 6-30-2, 13-30-2, 23-28-7, 28-30-2 which showed low BBTD incidence (<50%) despite the high aphid colony count the underlying mechanism could be resistance of the host to the virus.

The five mutant lines planted in multi-locations showed low incidences of the BBTV from planting to harvest. The highest incidence of the disease in the whole experimental area was recorded at 11% in Bay, Laguna trial site. The random mixture of mutant lines with varying resistance mechanisms could have prevented the rapid spread of the vector and virus in farmer's field. The farmer's practice of regular rouging of infected plants at least once a month within the trial sites prevented secondary infection. There was no intensive spread of the disease and the infection occurred at random.

Further studies on the use and deployment of resistant mutant lines as a component of BBTV management strategy are needed. The use of mutant multi line (mixture of mutant plants with different resistance mechanisms) as an approach for BBTV control needs further testing. Mass propagation and dissemination of BBTV-resistant mutant lines will make a tremendous impact on the banana industry. The use of BBTV- resistant mutant lines of bananas cv. Lakatan will offer the most effective control measure against the devastating BBTV disease.

3.1.4 Vietnam

The data showed that yield parameters of mutant lines were significantly different and higher than control line. The II-47 mutant line had a number of fingers is 18.65; otherwise, the control is 17.29. The yield estimate reached 32.75 tons/ha, meanwhile, control yield reached 30.56 tons/ha The mutant banana lines is being assigned by The Fruit and Vegetable Institute for their maintenance.

3.2 Conclusion and Suggestion

3.2.1 Bangladesh

In vitro regeneration protocol of *Fusarium* susceptible banana cv. Sabri (AAB) was established on MS medium supplemented with 5.0 mg/l BA + 2% Ads. *In vitro* radiosensitivity (LD_{50}) was determined at 35 Gy. About 2664 plantlets were transferred to the poly bags containing *Fusarium* infected soil and found heavily infested of plantlets and died after 2 to 3 months. About 1,000 Plants were transferred to the field with hot spot condition also showed symptoms of *Fusarium* and died within 6 to 8 months. Hence, further study needed to induce scalp and establish embryogenic cell suspensions for irradiation in order to develop disease resistance banana genotypes.

From the overall results of doubled haploid production from anther culture, it may be concluded that MS based medium is superior to N6 based medium in anther culture of banana. However, the frequency of anther response is very low. So it is needed to improve the efficiency of anther culture in banana by manipulating medium composition and/ or cultural conditions. The ultimate aim of this work is to regenerate plants from mutagenised gametic cells using anther culture of banana. So efforts will be made to regenerate plants from irradiated anthers.

3.2.2 Malaysia

In this project, meristems from shoot tips were used as explants or starting materials for mutation induction. Radiosensitivity test using a series of gamma ray doses of 0-100 Gy showed that LD50 for Pisang Berangan was 50Gy and selection for optimum doses based on shoot regeneration were 20, 30 and 40Gy. Four artificial disease screening techniques had been developed which can be used effectively for nursery and field screening.

1. Dipping method: 1-2 hrs soaking in spore suspension (10 6 spores/ml)
2. Double tray method: hardened plants for 4 weeks are planted in sterile sand media in tray and *Fusarium* spores are poured in the first tray.
3. Nursery screening method: Plants are hardened for 4-8 weeks in individual polybags and transfer in tray with coir dust containing *Fusarium* for 2 weeks. Evaluation is done 4-6 weeks after planting.
4. Field screening: Those that survived from the above screening method are transferred to hot spot. Resistant plants are multiplied and transferred back to hot spot for three generations.

From a total population of 1,115 irradiated plants which had been screened in the field, at present 3 potential mutant lines of Pisang Berangan tolerance to Fusarium wilt disease had been selected with improved traits such as high yield, early fruiting and short stature.

1. High yield - 20 Gy (high bunch weight, more than 30 kg/bunch)
2. Early flowering (9 months) - 20 Gy
3. Shorter tree - 40Gy

Other significant achievements

1. Collaboration with University of Malaya, private nursery (Syarikat Jalur Lipur), farmers and commercial company (United Plantations Sdn. Bhd.)
2. Supplying of tissue culture materials to nurseries and growers
3. Signing Memorandum of Agreement with commercial grower, Selamat Indah Sdn. Bhd. for the technology transfer of tissue culture technique for micropropagation of Pisang Berangan and commercialization of selected mutants

Suggest to carry out the following studies:

1. Molecular characterization of mutant lines.
2. Proposal for new related projects, such as study is to understand molecular responses demonstrated during the host-pathogen (*Fusarium oxysporum* and nematode) interaction, which presents an important step towards developing transgenic banana with survivability against Fusarium wilt and nematode infestations.

3.2.3 Philippines

Ten promising BBTV resistant mutant 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) were selected after three generations of continuous field evaluation under high BBTV infection. The reactions of the selected mutant lines to the aphid vector and BBTV and the possible underlying mechanism of resistance were studied. Resistance in some lines could be due to less preference of the vector to the mutant lines, while in other lines, it could be resistance of the host to the virus.

The low incidences of the banana bunchy top disease observed in multi-location trial sites despite the presence of virus and vector suggest that random mixture of several mutant lines with varying resistance mechanisms could offer a more sustainable control measure.

Mass propagation and dissemination of BBTV-resistant mutant lines will make a tremendous impact on the Philippine banana industry. The use of BBTV- resistant mutant lines (as mutant multi line) of bananas cv. Lakatan will offer the most effective control measure against the devastating BBTV disease. Prior to full commercialization of these mutant lines, there is need for a multi-location and demonstration trial in several banana growing areas for yield and agronomic performance and as means of technology demonstration and promotion to farmers, growers and interested entrepreneurs. Registration of mutant lines will also be made upon completion of the required data. A new project proposal to conduct multi-location trials and commercialization of these mutant lines was submitted to the PCARRD for funding. The mutant lines are maintained and micropropagated while waiting for support for commercialization.

3.2.4 Vietnam

Throughout all experiments, we had some conclusions: regeneration medium: MS + 0.5 ppm NAA + 3 ppm BAP, rapid multiplication medium: MS + 0.5 ppm NAA + 4 ppm BAP, root medium: MS + 0.5 ppm NAA + 0.2 ppm IBA. Gamma treatment had some variations: change in high, the color

of bud, leaf, and frequency variation from 1.8% to 12.5% at *in vitro* formula and from 7.5% to 15.7% at gamma mutagenic treatment formulas. We carried out artificial inoculation on banana in greenhouse. Spore density; 10^5 spores/ml, with 4 methods to taking inoculate in which, cultured hydroponic reveals high infected sensitive. We have obtained 17 banana lines with enhanced resistance to Foc disease in greenhouse (infected rates <25%).