

## INDUCING GENETIC VARIABILITY OF BLACK PEPPER (*Piper nigrum* L.) by $\gamma$ IRRADIATION

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### ABSTRACT

Genetic variability of black pepper germplasm in Indonesia is low. To broaden genetic variability, newly growth shoot tips from *in vitro* culture of black pepper var. LDL were  $\gamma$  irradiated with doses 0, 0.3 0.6, 0.9, 1.2 and 1.5 krad. The treatments were designed in a complete block with five replications. The irradiated plantlets were grown on MS medium. Response of the variety is described by recording an increase in leaves, shoots and node, numbers, plantlet height, and morphological abnormality in the first vegetative mutation generation (MV1) and the second vegetative mutation generation (MV2). After 6 weeks, the plantlets were sub cultured and the leaves of MV2 were used for RAPD analysis. Six random primers were used for the study, i.e. OPC-01 (TTCGAGC-CAG), OPC-02 (GTGAGGCGTC), OPC-04 (CCGCATCTAC), OPC-05 (GATGACCGCC), OPC-06 (GAACGGACTC) and Abi 117.17 (GCTC-GTCAAC). The results showed that the lowest averages value on the increase of leaves, shoots, nodes and plantlets height at MV1 are resulted at dose 1.5 krad, whereas dose 0.3 krad increases averages value on shoots and plantlet height. The highest percentage of abnormal leaves is resulted at dose 1.2 krad. After subculture, the MV2 plantlets showed higher averages value for almost all parameters observed than the untreated plantlets. The number of score able bands varied from 2-5 bands with molecular weight 0.4-12 kb. Thirty three bands were detected from the six primers, with OPC-01, OPC-04 and OPC-06 showed polymorphisms with 8 (24%) polymorphic bands. In OPC-01 one band with DNA size 1-1.5 kb was absence from the treated plants at dose 0.9-1.5 krad, while with OPC-04, one band size 1.5 kb present only at 1.2 krad and with OPC-06 one band size 12 kb absence from 0.6 and 0.9 krad, and 3-5 bands size 1.5, 1.8 and bands with size 3-12 kb disappeared at dose 1.2 and 1.5 krad. The appearance and disappearance of bands may be related to the genetic changes due to  $\gamma$  irradiation, and further exploration may be needed to find how much genetic variation induced by irradiation in field and the relationships with the changes in plant characters.

Key words: *Piper nigrum* L., mutation, irradiation, RAPD, genetic variation

### ABSTRAK

#### Peningkatan keragaman genetik tanaman lada (*Piper nigrum* L.) dengan iradiasi sinar gamma

Keragaman genetik plasma nutfah lada sempit, untuk meningkatkan keragaman genetik, mata tunas yang tumbuh dari biakan lada varietas LDL diradiasi dengan sinar  $\gamma$  dengan dosis 0, 0.3 0.6, 0.9, 1.2, dan 1.5 krad. Perlakuan menggunakan rancangan acak lengkap dengan lima ulangan. Tunas hasil radiasi ditanam pada media MS. Respon tanaman terhadap perlakuan iradiasi dilakukan dengan mengamati peningkatan jumlah daun, tunas, buku, tinggi tanaman dan morfologi pada planlet hasil perbanyakan vegetatif generasi pertama (MV1) dan kedua setelah iradiasi (MV2). Tunas hasil perbanyakan sub-kultur setelah iradiasi (MV2) dianalisa keragaman genetiknya dengan RAPD (Randomly Amplified Polymorphic DNA) menggunakan enam primer acak, yaitu OPC-01 (TTCGAGCCAG), OPC-02 (GTGAGGCGTC), OPC-04 (CCGCATCTAC), OPC-05 (GATGACCGCC), OPC-06 (GAACGGACTC) dan Abi 117.17 (GCTCGTCAAC). Hasil penelitian menunjukkan bahwa iradiasi menyebabkan perubahan yang nyata pada planlet generasi pertama setelah perbanyakan vegetatif (MV1) terutama pada jumlah buku dan tinggi tanaman, tetapi tidak berbeda nyata untuk penambahan jumlah daun dan

tunas. Nilai rata-rata penambahan jumlah daun, tunas, buku dan tinggi planlet terendah ditunjukkan oleh perlakuan iradiasi pada dosis 1.5 krad, sedangkan pada iradiasi 0.3 krad meningkatkan nilai rata-rata jumlah tunas dan tinggi planlet. Persentase daun abnormal diperoleh pada perlakuan 1.2 krad. Setelah sub-kultur, planlet generasi kedua setelah perbanyakan vegetatif (MV2) yang tumbuh menunjukkan nilai rata-rata yang lebih tinggi dari normal pada semua parameter. Persentase daun variegata pada MV1 diperoleh dari perlakuan 1.2 krad tetapi pada MV2 diperoleh dari perlakuan 0.6 krad. Jumlah pita DNA yang teramplifikasi berkisar antara 2-5 dengan berat molekul 0.4-12 kb. Tiga puluh tiga pita terdeteksi, 8 (24 %) pita diantaranya polimorfik, yang berasal dari primer OPC-01, OPC-04 dan OPC-06. Pada OPC-01 satu pita dengan ukuran 1-1.5 kb hilang dari perlakuan 0.9-1.5 krad, sementara pada OPC-04, satu pita dengan ukuran 1.5 kb muncul hanya pada perlakuan 1.2 krad dan pada OPC-06 satu pita 12 kb hilang dari perlakuan 0.6 dan 0.9 krad, 3-5 pita dengan ukuran 1.5 kb, 1.8 kb dan antara 3-12 kb hilang dari perlakuan 1.2 dan 1.5 krad. Hilang dan munculnya pita kemungkinan berhubungan dengan perubahan genetik akibat radiasi sinar  $\gamma$  dan penelitian lanjut perlu dilakukan untuk mengetahui tingkat keragaman yang ditimbulkan akibat iradiasi di lapang dan hubungannya dengan perubahan sifat terutama sifat yang menguntungkan.

Kata kunci: *Piper nigrum* L., Lada, mutasi, radiasi, RAPD, variasi genetik

### INTRODUCTION

Black pepper (*Piper nigrum* L.) is one of the most popular spice crops in the world. Black pepper was introduced to Indonesia from Malabar India and nowadays the crop has been cultivated in several places with the centre of productions in Bangka, Lampung and West Kalimantan. In Indonesia, this crop has a strategic role since it is almost 100% of all black pepper areas (136.145 ha of the total area of 136.450 ha) are cultivated by small farmers and become their sources of incomes. Indonesia market in the year 2000 reached 68% for white pepper and 24% for black pepper with export volume 65 000 tons and earns devise as much as 221 millions US\$ (BPS, 2000). Lampoeng black pepper and Munthok white pepper are the world's black pepper brand names from Indonesia.

The main constrains on black pepper production in Indonesia are low productivity and quality as well as wide spread of foot rot disease caused by *Phytophthora capsici* Linn. The pathogen caused crop lost up to 10-15% annually (KASIM, 1990). Crop improvement to obtain high quality, yield and disease resistance is hampered due to low genetic variability in the germplasm available in Indonesia.

Induction of genetic variability can be done through mutation. Mutagen treatment can increase mutant frequency drastically, and therefore are of outstanding importance for

breeding purposes (PREIL, 1986). Variations caused by induced mutations is not essentially different from variability caused by spontaneous mutations during evolution.

Mutation induction to increase genetic variability of black pepper has also been done in Malaysia and India. In Malaysia, mutation was limited to variety Kuching while in India, seven varieties has been induced; and for both countries seeds are used as plant materials (RAVINDRAN *et al.*, 2000).

Mutation can be induced by physical or chemical mutagens; both have been widely used in commercial breeding of many crops. To induce mutations in vegetatively propagated plants, chemical mutagens are not usually considered, mainly because they are not very successful, due to poor uptake and penetration of the chemical compounds (IAEA, 1986). For vegetative plant tissues, irradiation is more effective in producing mutants (BROERTJES, 1972).

Various types of ionizing radiation (X-rays, gamma rays, neutrons, ultraviolet light, etc) have been widely used in inductions of mutations in vegetatively propagated plants. Mutation induction on somatic cells using  $\gamma$  irradiation causes damages to cells, H<sub>2</sub>O molecules, DNA, enzyme, and growth regulators in plants (HANDRO, 1981). Ionic radiation such as  $\gamma$  irradiation could cause lethality, cytological damages, sterility and chimeras on first generation (IAEA, 1977). The genetic changes due to irradiation could increase genetic variability.

Worldwide, some of the recent advances in agriculture are linked to the success in both breeding and advances in *in vitro* techniques, particularly tissue culture. Mutation breeding carried out on selected crops such as vegetatively propagated crops has been made possible by combined application of mutation and *in vitro* technology. Artificial induction of somatic mutations through the used of ionizing radiation has proven to be an efficient technology for increasing the frequency of spontaneous mutations, which can lead to the development of new varieties.

By using combination of irradiation and *in vitro* technology, has led to the development of resistance of sugarcane to *Puccinia melanocephala* Butl. and *P. kuhnii* Butl. (NAGATOMI, 1996); resistance of ginger to *Ralstonia solanacearum* on early vegetative generations (HOBIR *et al.*, 1996), improve resistance of vanilla *in vitro* (MANGOEN-DIDJOJO *et al.*, 2000); and early maturity and new and improved germplasm in banana (MAK *et al.*, 1996).

The objectives of this study was to induce genetic variability of black pepper by using  $\gamma$  irradiation.

## MATERIALS AND METHODS

The research was conducted from January-December 2003 in the Plant Genetic Resources and Breeding Laboratory, ISMECRI, Bogor.  $\gamma$  irradiation was undertaken in the Center for Research and Development for Isotope and Radiation Technology, National Atomic Energy Agency, Pasar Jumat, Jakarta Selatan.

### Mutation Induction

Single node explants of black pepper var. LDL (Lampung Daun Lebar) were cultured *in vitro* on MS medium (MURASHIGE and SKOOG, 1962) added with BAP 0.3 mg/l (SUKMADJAJA, 1992). Newly growth shoot tips were transferred into the fresh medium. One month after sub-culture the explants were irradiated with  $\gamma$  rays at dose 0 (control), 0.3, 0.6, 0.9, 1.2, and 1.5 krad, with 2,252188081 kGy/h (dose 1 kGy or 100 krad was obtained by irradiation for 1590 seconds). The experiment was designed in a complete block design with 5 replications. Each replication consists of eight explants. Their plantlets were incubated at temperature 22-25°C, with light intensity 1000 lux for 16 h/d. Observations were undertaken every 2 weeks until 6 weeks after irradiation on the newly growing shoots that comes from the base of the leaf petiole (the first vegetative mutation generation after irradiation (MV1). After 6 weeks, newly growing shoot tips were cut and sub-cultured onto the fresh medium and becomes the second vegetative mutation generation after irradiation (MV2). Observation was made every two weeks until 8 weeks after culture. Parameters observed on MV1 and MV2 were number of leaves, shoots, nodes, plant height, abnormal leaves, abnormal plantlets, plantlet performance (leaves colour and shapes, length of internodes).

### Genetic Analyses

Leave samples of the treated plantlets from MV2 were analyzed for their DNA changes using RAPD methods. DNA was extracted from leaves based on a modified method of OROZCO-CASTILLO *et al.* (1994) added with PVPP (polyvinyl poly pyrrolidone) and mercapto-ethanol. DNA was purified using a mixture of Chloroform and iso-amylalcohol (24:1). DNA concentration was determined using minigel (SAMBROEK *et al.*, 1989) with  $\lambda$  DNA as a standard or UV spectrophotometer at wavelengths A260 and A280. Six random primers were used for the study, i.e. OPC-01 (TTCCAGCCAG), OPC-02 (GTGAGGCGTC), OPC-04 (CCGCATCTAC), OPC-05 (GATGACCGCC), OPC-06 (GAACGGACTC) (Operon

Technology, USA) and Abi 117.17 (GCTCGTCAAC) (Australia). 25  $\mu$ l of mixture containing 50 ng genomic DNA, primers, Tris-HCl, KCl, MgCl<sub>2</sub>, dNTPs and *Taq* polimerase are amplified on *Thermal Cycler*. Electrophoresis was performed on agarose gel 1.4% TAE buffer, stained with Ethidium bromide.

## RESULTS AND DISCUSSION

### Mutation Induction

Visually, the initial growth of the MV1 plantlets after irradiated showed good performance; no intoxicated symptoms was noticed. Effect of the treatment was significant on the MV2 plantlets after sub-cultured (Figure 1). The treated plants produced more leaves than its control. The highest increase in average leaf number was observed from the treatment 1.2 krad after 2 to 4 weeks sub cultured, while at 6 – 8 weeks the highest leaves was obtained from treatment at 1.5 krad.

Irradiation also caused changes in leaves shape, size and colour. At doses 1.2 krad and 1.5 krad leaves were light green to yellowish green and the shapes and size vary some have smaller leaves. At lower doses the leaves colour and shapes were almost similar to control. This indicated that higher irradiation doses affected leaves shape, size and pigmentations. Eight weeks after subculture, the leaves of the MV2 plantlets treated at doses 0.6 and 0.9 krad were greener than control, while from doses 1.2 and 1.5 krad the leaves turn its colour to green as normal. This seems that the plants were able to recover from the irradiation.

Although, shoots formation was not significantly affected by irradiation (Figure 2), it was noticed however, that lowest average shoot number was obtained from treatment at dose 1.5 krad. Irradiation slowed down shoot formation at higher dose. The highest increase in shoot formation in after subculture (MV2) was obtained from treatment at 1.2 krad. ICHIKAWA and IKUSHIMO (1967) stated that irradiation could inhibit shoot formation, but in some cases high irradiation dose promote shoot formation. There is dosage specificity of each species to induce shoot

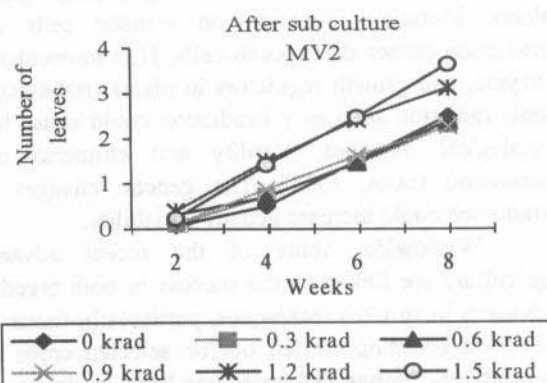
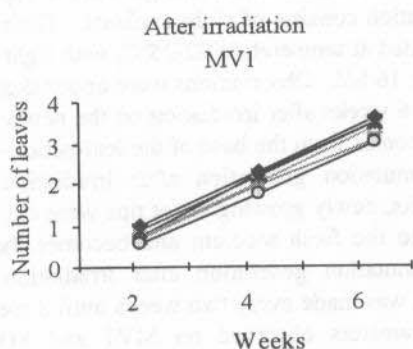


Figure 1. The effect of irradiation dose at average leaves number on MV1 and MV2 plantlets  
Gambar 1. Pengaruh dosis iradiasi terhadap rata-rata jumlah daun pada plantlet MV1 dan MV2

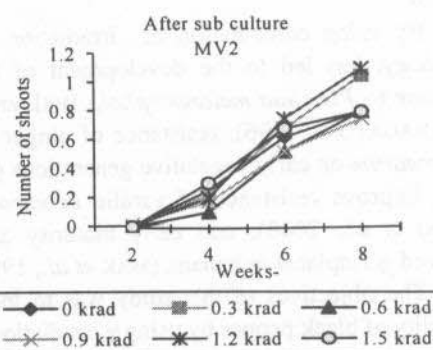
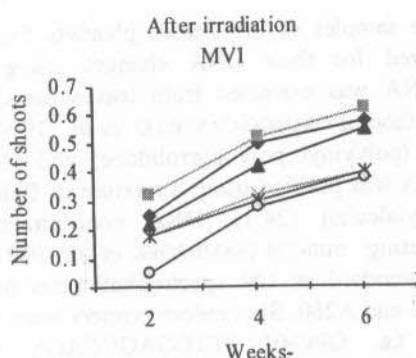


Figure 2. The effect of irradiation dose on the increase in shoot number  
Gambar 2. Pengaruh dosis iradiasi terhadap penambahan jumlah tunas



formation. Irradiation at 100 rad promoted shoot formation in *Anthurium andreaeanum* (PIERIK, 1987), while in gerbera shoot formation increase at irradiation dose 500 rad (PRASETYORINI, 1991).

Irradiation significantly affect the number of node at 4 and 6 weeks after irradiation (Figure 3). The highest number of nodes increased was shown by control. The higher doses inhibited nodes formation. After sub culture, the highest increase in node number was shown at dose 1.2 krad. This might indicate that irradiation at 1.2 krad accelerate the node formation in black pepper.

The internodes length after irradiation at 1.2 and 1.5 krad were shorter than those at lower doses, and cause plantlets to become dwarf and rosettes. At lower doses the length of internodes are similar to control.

Irradiation also affect plantlet height significantly at 1.5 krad (Figure 4). Four and six weeks after culture, plantlets from treatment with irradiation at doses 1.5 krad was very short, with small leaves, while at dose 0.3 krad,

irradiation increased plant height. It indicated that irradiation damaged meristematic cells at the shoot apex which affect shoot elongation. Shoot elongation was influenced by IAA production in the shoot apex. Irradiation inhibited IAA production in the shoot apex as a result of reduction in the synthesis of Indolacetaldehyde dehydrogenase enzyme which is radiosensitive (ICHIKAWA and IKUSHIMO, 1967; GROSCH and HOPWOOD, 1979). BROERTJES and VAN HARTEN (1988) stated that meristematic cells such as those of cells in shoot apex are more radiosensitive than older cells. High irradiation dosage could inhibits the growth of the meristematic cells. After sub-culture, plantlet height improved especially on treatment at 1.2 krad. Apparently, transferring into fresh medium with fresh endogenous hormones, the plantlets could recover and grow better for those treated with 1.2 krad, but the damage on the 1.5 krad was to high so that sub-culturing of the plant did not improve plant growth.

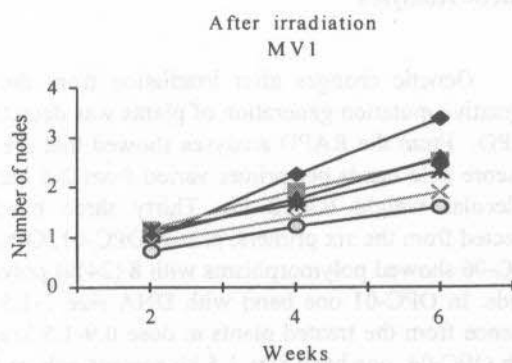


Figure 3. The effect of irradiation dose on the increase in node number  
Gambar 3. Pengaruh dosis iradiasi terhadap peningkatan jumlah buku

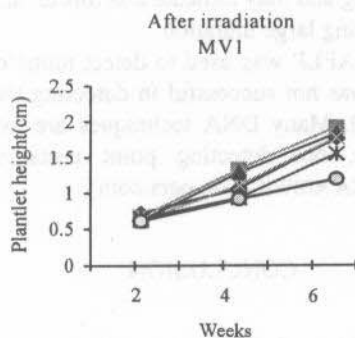
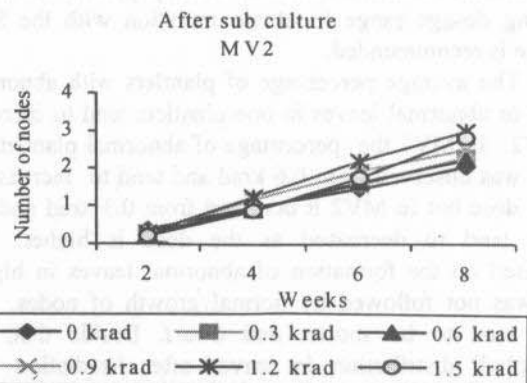
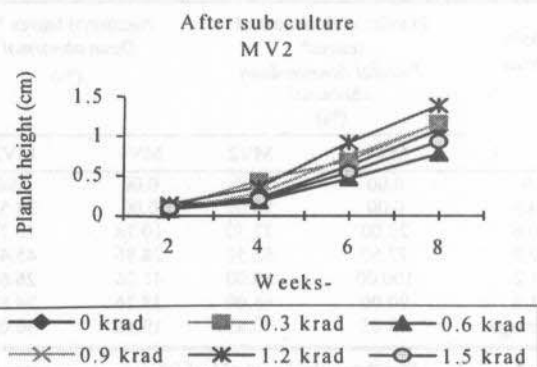


Figure 4. The effect of irradiation dose on plantlet height  
Gambar 4. Pengaruh dosis iradiasi terhadap tinggi plantlet



Morphological changes were observed on plantlets after irradiation (MV1) and after subculture (MV2). The higher irradiation dose the higher abnormality on morphology were obtained. At doses 0.3 and 0.6 krad, irradiation caused abnormality on leaves shape and size. At higher doses 0.9, 1.2, and 1.5 krad, beside abnormality on leave shape, the internodes also become short and the shoots rosettes. Although, the highest percentage of abnormal leaves on MV1 is found at dose 1.2 krad, but on MV2 is resulted from dose 0.6 krad (Table 1). More plantlets from the 1.5 krad were dwarfs. Physiological damage on cells due to irradiation lead to abnormal development of plantlets. GROSCH and HOPWOOD (1979) indicated that irradiated plants showed growth abnormality in terms of dwarf ness, shape, thickness, texture, leaves shapes, veins, midrib, colour, chlorophyll distribution. This seems that reduction or inhibition in certain cells might induce better growth in other cell parts. Differences of cells radio sensitivity following irradiation may lead to differences in the level of morphological abnormality. Based on the results, 1.5 krad in black pepper is too high, reducing dosage range to obtain mutation with the least damage is recommended.

The average percentage of plantlets with abnormal leaves or abnormal leaves in one plantlets tend to increase in MV2. In MV1 the percentage of abnormal plantlets or leaves was observed from 0.6 krad and tend to increase in higher dose but in MV2 it occurred from 0.3 krad and the values tend to decreased as the dose is higher. The decreased on the formation of abnormal leaves in higher dose was not followed by normal growth of nodes. The nodes tend to be shorter and dwarf. Beside that, the chlorophyll distribution in leaves after irradiation was abnormal. Physiological changes in chlorophyll synthesis

which eventually deficiency in chlorophyll was noticed by showing lead to variegated. The highest percentage of variegated leaves on MV1 was obtained on 1.2 krad but on MV2 was at 0.6 krad. The changes in the percentage on variegated leaves to lower dosage may be related to the ability of the cells to recover and repair the synthesis of chlorophyll. Similar results were also obtained in India,  $\gamma$  irradiation on black pepper seeds caused adverse affect on germination of seeds, as the dose increased germination was delayed (RAVINDRAN *et al.*, 2000). The M1 population showed certain abnormalities such as chlorophyll changes, twinning of seedlings and rosette of leaves. The frequency of chlorophyll abnormalities in the M1 population was 0.1-13% depending on the radio-sensitivity of the varieties. Chlorophyll abnormalities recorded in black pepper was albino, xantha (yellow) and variegated. In other plants such as in *Bougainvillea*, most of variegated leaves were found after irradiation at 7.5 Gy, while at 10 Gy all leaves are abnormal (BANERJI *et al.*, 1987).

### Genetic Analyses

Genetic changes after irradiation from the second vegetative mutation generation of plants was detected using RAPD. From the RAPD analyses showed that the number of score able bands per primer varied from 2-5 bands with molecular weight 0.4-12 kb. Thirty three bands were detected from the six primers, primer OPC-01, OPC-04 and OPC-06 showed polymorphisms with 8 (24%) polymorphic bands. In OPC-01 one band with DNA size 1-1.5 kb was absence from the treated plants at dose 0.9-1.5 krad, while with OPC-04, one band size 1.5 kb present only at 1.2 krad and with OPC-06 one band with molecular weight 12 kb absence from 0.6 and 0.9 krad, and 3-5 bands sizes 1.5 kb, 1.8 kb and bands with size between 3-12 kb disappeared from the plants treated at dose 1.2 and 1.5 krad (Figure 5). The ability to detect DNA changes after  $\gamma$  irradiation using RAPD is amazing and may indicate that this techniques was reliable in detecting large mutation

In roses, AFLP was used to detect mutation after X irradiation but was not successful in detecting the changes (IBRAHIM, 1999). Many DNA techniques are available to detect mutation, but detecting point mutation mostly difficult (KARTIKA ADIWILAGA, pers.com).

### CONCLUSION

Combination of irradiation and *in vitro* technique were effective in producing genetic variation in black pepper. The applied doses of irradiation (0.3 – 1.5 krad) induced changes on morphological characters of the plantlets on both MV1 and MV2 generations. Irradiation

Table 1. Percentage of abnormal plantlets and leaves at MV1 and MV2  
Tabel 1. Persentase plantlet dan daun abnormal pada MV1 dan MV2

Dose Dosis	Plantlet with abnormal leaves* Plantlet dengan daun abnormal (%)		Abnormal leaves ** Daun abnormal (%)	
	MV1	MV2	MV1	MV2
0	0.00	0.00	0.00	0.00
0.3	0.00	50.00	0.00	34.58
0.6	25.00	72.50	10.78	48.74
0.9	77.50	62.50	28.86	45.44
1.2	100.00	55.00	41.26	26.60
1.5	90.00	65.00	38.26	24.82
Average	43.25	50.83	19.86	30.03

Note : \* Plantlet with abnormal leaf (s)

\*\* Number of abnormal leaves in one plantlet/total number of leaves

Keterangan : \* Plantlet dengan daun abnormal

\*\* Jumlah daun abnormal dalam satu plantlet/jumlah daun

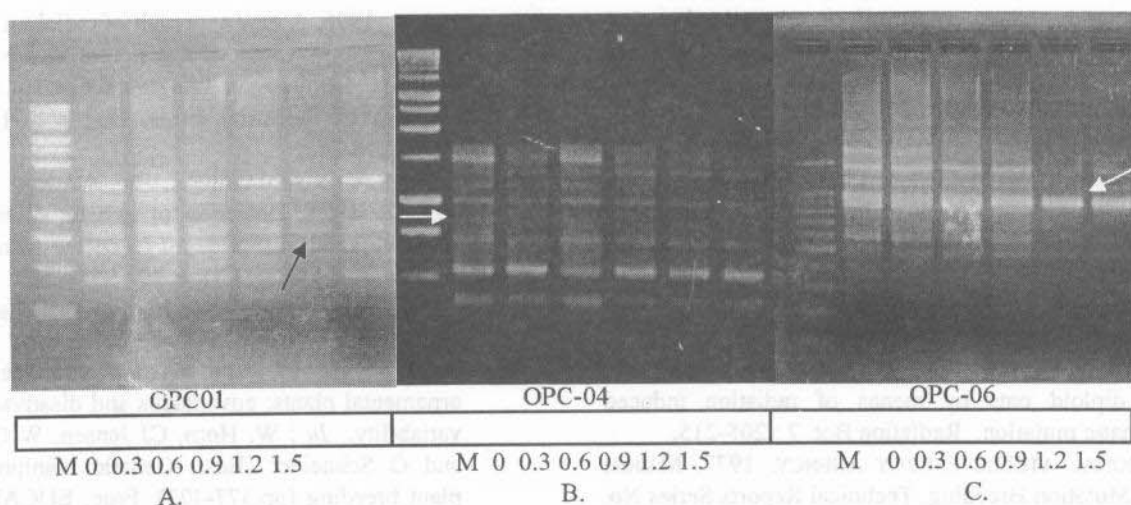


Figure 5. RAPD patterns of irradiated black pepper plantlets. (M- $\lambda$  DNA as marker, A. An arrow indicated the presence of a band in 1.2 krad but absence in other treatment. B. A white arrow indicated the presence of smear band in 0-0.6 krad but absence from the other treatment and C. A white arrow indicated the absence of 2-5 bands from the 1.2-1.5 krad while in the other the bands are present)

Gambar 5. Pola RAPD pada plantlet lada hasil iradiasi (M- $\lambda$  marka DNA, A anak panah menunjukkan adanya pita baru pada perlakuan 1.2 krad. B. Panah menunjukkan adanya pita pada perlakuan 0-0.6 krad tapi hilang dari perlakuan lainnya dan C. Panah menunjukkan hilangnya 2-5 buah pita dari perlakuan 1.2 – 1.5 krad)

dose at 1.5 krad, produced plantlets with the lowest value on the increase of leaves, shoots, nodes and plantlets height. This treatment also produced plantlets with the highest percentage of dwarfs whereas dose 0.3 krad increases averages value on shoots and plantlet height. While the highest percentage of abnormal and variegated leaves was obtained from treatment 1.2 krad. Sub-culture of MV1 shoots, showed even higher changes for almost all parameters observed compared to the untreated plantlets.

Thirty three bands were detected from RAPD analysis on MV2 plantlets, 8 (24%) bands were polymorphic. Primer OPC-01, OPC-04 and OPC-06 showed polymorphisms. Several bands were absences from the treated plantlets i.e. one band with DNA size 1-1.5 kb absent from the treated plants at dose 0.9-1.5 krad (OPC-01); one band size 12 kb absence from 0.6 and 0.9 krad, and 3 to 5 smear bands size 1.5 kb-1.8 kb and bands with size 3-12 kb disappeared from plants treated at dose 1.2 and 1.5 krad (OPC-06). One new band size 1.5 kb present only at 1.2 krad detected with OPC-04.

The appearance and disappearance of bands may be related to the genetic changes due to  $\gamma$  irradiation. At least four new variants were observed from this study. Further exploration may be needed to find the relationships between the appearance and disappearance of bands with the changes in plant characters especially on changes in the favorable characters.

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