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ABSTRACT

Mutation breeding has become a proven method of improving crop varieties. Most research on plant mutation breeding in Indonesia is carried out at the Center for Research and Development of Isotope and Radiation Technology, National Nuclear Energy Agency (BATAN). Nowadays, a biotechnological approach has been incorporated in some mutation breeding researches in order to improve crop cultivars. This approach is simply based on cellular totipotency, or the ability to regenerate whole, flowering plants from isolated organs, pieces of tissue, individual cells, and protoplasts. Tissue culture technique has been extensively used for micro propagation of disease-free plants. Other usage of this technique involves in various steps of the breeding process such as germplasm preservation, clonal propagation, and distant hybridization. Mutation breeding combined with tissue culture technique has made a significant contribution in inducing plant genetic variation, by improving selection technology, and by accelerating breeding time as for that by using anther or pollen culture. In Indonesia, research on mutation breeding combined with tissue culture techniques has been practiced in different crop species including rice, ginger, banana, sorghum etc. Specially in rice, a research on identification of DNA markers linked to blast disease resistance is now still progressing. A compiled report from some research activities is presented in this paper.

INTRODUCTION

By definition, biotechnology is the application of a number of techniques and tools of cell biological and molecular biological origin in crop plants for economic purposes (Van Harten, 1998). Such techniques may help us to better understand genetic processes and be of value for plant breeding research as well as directly utilized for the production of new, genetically improved cultivars. It was also noted by Van Harten (1998) that basically there are two groups of biotechnological activities can be distinguished in plants. The first group is all activities related to in-vitro culture and the second group is the various recombinant DNA methods. Techniques belonging to both groups are often combined and several ways of using recombinant DNA would not even possible without having suitable in-vitro systems.

Basically, in-vitro culture refers to growing different plant organs, explants, tissues, cell suspensions, single cells, and protoplasts under control and aseptic conditions. Application of in-vitro methods has become practice in many recent mutation breeding projects. In Indonesia, for instance, tissue culture techniques have been

practiced to generate mutant lines of several crop species including rice, ginger, banana, sorghum etc.

The most important recombinant DNA technology is genetic transformation which implies the transfer and incorporation of foreign DNA into the DNA of a recipient plant cell, followed by recombination of that DNA into the cell's genome. For useful application of this technique in plant breeding, it is essential that the newly integrated foreign DNA is expressed in a controlled way in the recipient cell and that transformed cells can be properly regenerated.

The application of recombinant DNA technology in plant mutation breeding has not been practiced yet in Indonesia. Anyhow, research on the application of other DNA technology i.e. the use of DNA markers for helping selection in a mutation breeding program has been initiated in rice. This research is now still under state of identifying DNA markers linked to blast disease (*Pyricularia grisea* Cav.) resistance in rice. Some experimental results including both the application of in-vitro techniques in plant mutation breeding programs and the preliminary research on identification of DNA markers are summarized in this paper.

MUTATION INDUCTION IN GENERAL

Plant breeding requires genetic variation of useful traits for crop improvement. Often, however, desired variation is lacking. Mutagenic agents, such as radiation and certain chemicals, then can be used to induce mutations and generate genetic variations from which desired mutants may be selected. Mutation induction has become a proven way of creating variation within a crop variety. It offers possibility of inducing desired attributes that either cannot be found in nature or have been lost during evolution. When no gene or genes for resistance to a particular disease or for tolerance to stress can be found in the available gene pool, plant breeder have no obvious alternative but to attempt mutation induction.

Treatment with mutagens alters genes or breaks chromosomes. Gene mutations occur naturally as errors in deoxyribonucleic acid (DNA) replication. Most of these errors are repaired, but some may pass the next cell division to become established in the plant offspring as spontaneous mutations. Spontaneous mutations happened in nature with a very slow rate. Induced mutation by using a certain mutagen has been proven to be able to speed up mutation in plants and it has become an alternative tool in plant breeding for increasing plant genetic variability.

Artificial induction of mutations by ionizing radiation dates back to the beginning of the 20th century. But it took about 30 years to prove that such changes could be used in plant breeding. Initial attempts to induce mutations in plants mostly used X-rays, but later on gamma and neutron radiation were employed as these types of ionizing radiations became readily available from newly established nuclear research centers.

BIOTECHNOLOGY IN PLANT BREEDING

Breeding for improved plant cultivars is based on two principles i.e. genetic variation and selection. The process is extremely laborious and time consuming with high inputs of intellectual and manual work. However, the development of plant cell and tissue culture has made it possible to transfer part of the breeding work from field to laboratory conditions.

Extensive research has resulted in a new areas of plant breeding namely "plant biotechnology" and "gene engineering". They are based on cellular totipotency or the ability to regenerate whole, flowering plants from isolated organs (meristems), pieces of tissue, individual cells, and protoplasts. The isolated plant parts are aseptically grown in test tubes on artificial media of known chemical composition (*in vitro* culture). Under strictly controlled conditions, they form plantlets that subsequently can be transferred to soil where they grow to maturity.

In plant breeding, tissue culture techniques have been exploited commercially for micro propagation of disease-free stocks of horticultural crops such as strawberry, potato, banana, ornamentals etc. *In vitro* techniques are also used in various steps of the breeding process, such as germplasm preservation, clonal propagation, and distant hybridization (Van Harten, 1998).

Radiation mutation breeding and isotope techniques, combined with tissue culture, have made a significant contribution to plant breeding. They have introduced new techniques for inducing genetic variation, by improving selection technology, and by accelerating breeding time (Novak and Brunner, 1992).

Other methods, known as anther or pollen culture, make it possible to regenerate plants from male gametes with half the number of chromosomes (haploids). Compared to plants with full chromosomal content (diploids), the use of haploids in mutation breeding is advantageous since it allows detection of mutations immediately after their induction. Haploid methods have proven to significantly speed up the breeding of new varieties of rice, barley, and vegetables (Novak and Brunner, 1992).

Genetic engineering procedures allow the transfer of genetic material (DNA) from the cell of one species to that of another genetically unrelated organism. For example, a piece of DNA from a bacterial cell may be integrated into the genome of a plant cell to form a transgenic plant. The new DNA (gene) expresses itself in the plant phenotype regenerated from the transgenic cell. Nuclear techniques, based on nucleic acid bases labeled by isotopes, are employed in genetic engineering, to identify and isolate suitable genes for transfer, as delivery system to introduce genes into recipient cells, and to detect new genetic material in recipient organisms. Genetic engineering already has resulted in the production of plants with new desirable traits, such as insect resistance, virus disease resistance, and better ripening properties. However, early enthusiasm is being tempered by the growing discussion of the potential hazards of releasing transgenic plants to the environment (Novak and Brunner, 1992).

MUTATION BREEDING IN INDONESIA

Mutation breeding in Indonesia was started in rice in 1972, initially in an attempt to improve protein content of rice grain (Ismachin and Hendratno, 1972). This research activity was carried out under the IAEA Research Coordination Program. In 1974, rice mutation breeding activities were focused on searching mutant lines resistant to brown plant hopper (BPH) because, at that time, serious epidemics were recorded in some important rice-producing areas in Indonesia. Following subsequent selection process for resistant plants, a number of mutant lines resistant to BPH were obtained (Mugiono, 1990). Some of the promising mutant lines were then tested for their yield potential at several locations.

In 1982, rice mutant line No. 627/4-214/Psj and 627/10-3/Psj were submitted for official release by the Department of Agriculture. However, only the former was received approval for release as a new variety by the name of *Atomita-1*. This new variety had a better grain quality, early maturing, high yield, resistant to BPH biotype-1 and green leaf hopper. Unfortunately, *Atomita-1* was not resistant to BPH biotype-2 so that this variety was not grown widely in the country because of the endemic situation of BPH biotype-2 in the rice field.

Following *Atomita-1*, other new mutant varieties of rice by the name of *Atomita-2*, *Atomita-3*, and *Atomita-4* were subsequently released by the Department of Agriculture (Mugiono and Hendratno, 1995). However, these mutant varieties were not grown widely by farmers. It was suspected that the reasons might be due to naming "*Atomita*" gave a frightening impacts to farmers who might think the mutant varieties contained a "dangerous atom" inside them. Therefore, since 1992, naming a new mutant variety for rice had been proposed to use a river name instead of *Atomita*, and it did give a significant impacts to farmer's acceptance.

It was proven by the release of new mutant variety of upland rice with the name of *Situgintung*. Beside resistant to BPH biotype-1 and biotype-2 and blast disease, this variety had high yield under upland condition. In 1996, another new mutant variety "*Cilosari*" was released by the Department of Agriculture. This variety was suitable for irrigated areas or lowland condition in Indonesia (Mugiono, 1997). At present, *Situgintung* and *Cilosari* are popular as they are now grown widely by farmers in almost all provinces in Indonesia.

Beside in rice, research on mutation breeding in Indonesia had also resulted some new mutant varieties of soybean and mungbean (Soeranto et. al., 2000). Three mutant varieties of soybean by local name of *Muria*, *Tengger*, and *Meratus* and one mutant variety of mungbean by the name of *Camar* had officially been released by the Department of Agriculture. These mutant varieties were also grown widely by farmers in several provinces in Indonesia. Meanwhile, some promising mutant lines of other crop species including peanuts, banana, onions, ginger, patchouly, and sorghum were still under field investigation for official release as new cultivars.

COMBINED BIOTECHNOLOGY AND MUTATION BREEDING

1) Tissue Culture Techniques in Ginger

Ginger plant (*Zinger officinale* Rosc.) is an important spice crop which demand is always increasing year by year. In Indonesia, ginger is generally used in various medicinal and culinary preparation in most local communities. Ginger is vegetatively propagated through the underground rhizomes but, unfortunately, its multiplication rate is very low. Hosoki et.al. (1977) reported heavy losses in ginger production in some plantations due to some disease attacks caused by bacterial wilt (*Pseudomonas solanacearum*) and soft rot (*Pythium aphanidermatum*). Because the diseases are mainly transmitted by rhizomes propagated every year, a production of disease-free clones are necessitated in order to get a successful ginger cultivation. Micro propagation by using tissue culture techniques can be a proper alternative to produce disease-free clones of ginger plant.

Problems faced in ginger breeding has so far been the very low genetic variation in ginger plant. This is because ginger is vegetatively propagated crop and hybridization is not effective since its flower biology has not been properly observed yet. Wide genetic variation is needed in plant breeding in order to search ideal plant types during the process of selection (Simmonds, 1986). In a breeding for disease resistance, the narrow genetic variation will slow down the selection process due to the risk of susceptibility to the disease.

A choice to increase plant genetic variability can be through somaclonal variation derived from tissue culture techniques, or through induced mutation techniques. Larkin (1981) stated that the somaclonal variation was a genetic variation produced from a tissue or cell cultures. Meanwhile, Reisch (1983) mentioned that somaclonal variation happened in differentiation stage of tissue or cell cultures can be increased by applying either chemical or physical mutagen.

A research on induced mutation in ginger plant combined with tissue culture techniques has been conducted at BATAN. The explants used were the shoot tips sized 0.4 - 0.5 cm long taken from the rhizomes. The explants were exposed to gamma rays emitted from Cobalt-60 source with different level of doses. Gamma chamber is available at BATAN with initial activity of about 10980 curries. The irradiated explants were then sterilized with 70 % alcohol and 0.2 % $HgCl_2$, rinsed 5 times with distilled water, plated in the modified MS basal media, and subcultured every 4 weeks. After 8 weeks, observations were done for the variable survival growth rate, number of shoot emerged, shoot height, and abnormality. Results of the observations were presented in Table 1.

As shown at Table 1, the best growth performance of ginger explants was for those irradiated with Gamma rays with the dose of 9 Gy. It seemed that dose inhibited bacterial and fungal contamination and stimulated explant growth. Lower doses as to 3 and 6 Gy did not show any better growth performance and it might be due to bacterial and fungal contamination. The Gamma ray doses higher than 9 Gy seemed to cause significant abnormalities as shown by dwarf plant with pale leaves. Based on

Table 1, data analysis for best fitting models found the relationship between Gamma ray irradiation doses with the survival rate follows the Sinusoidal equation i.e. $Y = 69.14 + 40.33 \cos(1.33X + 0.48)$ with coefficient of regression $r = 0.921$.

Table 1. The effects of Gamma rays on ginger tissue culture after 2nd subcultured.

Gamma rays doses (Gy)	Survival rate (%)	Number of shoots	Shoot height (cm)	Abnormality
0	85.0	2-7	1.5-8.0	None
3	62.5	4-8	1.5-8.0	None
6	43.8	2-8	2.0-8.0	None
9	87.5	2-16	1.5-9.0	None
10	85.0	2-6	1.5-6.0	Dwarf, pale leaf
12	25.0	2-3	1.5-5.0	Dwarf, pale leaf
15	50.0	2-5	1.0-5.0	Dwarf, pale leaf
20	37.5	1-2	1.0-2.0	Dwarf, yellow leaf

Modification in MS basal media with thidiazuron (TDZ) hormone significantly improved explant growth as shown in Table 2 (Ismiyati et. al., 2000). Following the next subcultures, plantlets were transplanted to the field for screening against bacterial wilt (*Pseudomonas solanacearum*) and soft rot (*Pythium aphanidermatum*) diseases.

Table 2. Growth performance of ginger explant culture in modified MS basal media with TDZ hormone.

Gamma ray doses (Gy)	Average			
	Explant growth (%)	No. of plantlets/explant	No. of leaves/plantlet	Plantlet height (cm)
0	100	3.3	3.0	5.9
6	80	2.6	3.0	6.9
8	100	2.5	2.7	6.9
10	97	2.8	3.5	6.7
15	92	3.7	3.1	7.0
20	86	2.1	3.4	6.4

2) Tissue Culture Techniques in Banana

Banana is an important fruit crops, ranked as second most important fruit in the world after citrus (Swennen & Rosales, 1994). The world production of banana in 1993 reaches 74 million tons (FAO, 1993). Banana production is concentrated in Central and South America, Africa, and Asia-Pacific with their contribution to the world production of 36, 35, and 29 %, respectively.

In Indonesia, the majority of edible banana is cultivated around the backyard with high soil fertility. In a country with densely increased population, like Indonesia, it is difficult to maintain banana cultivation around the backyard. Shifting its cultivation to another large-scale arable land may also compete with annual food crops or with other horticultural crops. Thus, banana production in the arable land is low and insignificant.

Banana is traditionally propagated by separating the suckers from their mother plant, which is then planted individually to establish a new mother plant. Because of that nature of propagation system, and the fact that banana flowers are mostly sterile, breeding banana by mean of hybridization is difficult to be conducted. Induced mutation combined with tissue culture techniques can be used as an alternative to increase plant genetic variation in a breeding program. Subsequent breeding procedures through selection processes can theoretically shorten breeding time of banana to reach the desired objectives.

Almost banana cultivars grown in Indonesia are susceptible to a plant disease caused by *Fusarium oxysporum* Cubense (FOC). Mutation breeding for this disease resistance has been conducted in combination with tissue culture techniques (Ismiyati et. al., 1998). A local banana variety *Ambon Kuning* (cavendish type) was used as the plant material. Its plantlets with height of about 5 cm were irradiated with Gamma rays emitted from Cobalt-60 source with the dose levels of 5-35 Gy. The irradiated plantlets were grown in MS basal media modified with adding growth regulators 4 ppm BAP and 2 ppm IAA. The grown plantlets were then subcultured up to M_1V_5 generation, acclimatized, and transferred to a hot spot field for *Fusarium* disease. Observations were done for plantlet performances during in-vitro stage and severity of *Fusarium* attacks in the field. Results of the observations were presented in Table 3, 4, and 5.

Table 3. Number of survival plantlets at different plant age after Gamma rays irradiation treatments.

Gamma ray doses (Gy)	Number of survival plantlets			
	3 months	6 months	9 months	12 months
0	312	423	511	554
5	324	414	482	538
10	272	347	392	468
15	275	351	412	471
20	94	0	0	0
25	96	0	0	0
30	78	0	0	0
35	45	0	0	0

Table 4. Plant performances in the field at 3 months after transplanting.

Gamma ray doses (Gy)	Average		
	Plant height (cm)	No. of leaves	No. of suckers
0	69.17	12.3	1.56
5	88	13.82	2.65
10	87.51	12.61	2.39
15	88.75	11.4	1.82

Table 5. Number of healthy and infected plants at 6, 7 and 8 months after transplanting in the field.

Gamma ray doses (Gy)	6 months		7 months		8 months	
	Healthy	Infected	Healthy	Infected	Healthy	Infected
0	19	31	16	34	8	42
5	12	38	9	41	7	43
10	25	25	22	28	15	35
15	35	15	34	16	28	22

3) Tissue Culture Techniques in Sorghum

Indonesia has so far been very much dependent on rice as a staple food. In order to guarantee food safety in the country, it is necessary to campaign food diversification utilizing other food resources available. Sorghum plant (*Sorghum bicolor* L.) is thought of among the ones need to be considered in attempt to make the food diversification program be successful. Sorghum's potential is primarily due to its high production, low-input crop (required less fertilizer), more tolerant to drought and other adverse conditions, and its high nutrition values. In many countries, sorghum has been used as either food, feed, or industrial crops. According to FAO (1994) sorghum is the world's fifth food after wheat, rice, corn and barley. As a food crop, sorghum was reported to have good nutrition values (Direktorat Gizi DEPKES RI, 1992). Unfortunately, sorghum has so far been grown very limited by Indonesian farmers who are used to grow rice in their lands. This limitation may be due to some problems such as availability of qualified seeds of sorghum varieties.

Farmers usually grow local variety in multiple cropping system with other crops where sorghum is used as an alley between crops. Normally, farmers use uncertified seeds of their own because certified ones are not available in the local market. From agronomic point of view, the local variety has some disadvantages as its characters of having high stature, late maturity, uninteresting seed color (brown), dropped head, and low production. The character of having high stature tends to make this variety be susceptible to lodging, and that of brown color is less desirable than that of the white one. For being used directly as food or in a food industry, white sorghum is usually more preferable.

In Indonesia, sorghum breeding was aimed at improving the local varieties for suitable use as food, feed, or industrial purposes. Unfortunately, variability of sorghum genotypes found in Indonesia is quite narrow and, therefore, different methods of breeding need to be operated. Meanwhile, Some improved sorghum lines as results of breeding by using mutation techniques have been produced, released and listed as new varieties (Wang, 1998 and Maluszynski et. al., 2000). Combining tissue culture techniques with induced mutations was one method that has been conducted at BATAN.

Seeds of sorghum varieties *Keris*, *Durra*, and *Ethio-95* was irradiated with different dose levels of Gamma rays emitted from Cobalt-60 source. The embryo was then separated from the endosperm and used as an explant in a tissue culture technique. The irradiated embryos were sterilized with 70 % alcohol and 0.2 % $HgCl_2$, rinsed 5 times with distilled water, plated in the modified MS basal media, and subcultured after 4 weeks. Plantlets resulted from subculturing were transferred to polybags and grown in greenhouse for producing seeds. All the seeds produced were grown in the field for selections. Plant selections were simply based on visually phenotypic performance of important agronomic traits such as plant height, plant age, head size, head form, seed size, seed color, productive tillers, and yield. Selected plants were grown in the next subsequent generations for further selections if they were still segregating. The promising mutant lines were then tested against drought in a drought-prone area i.e. in Gunung Kidul District of Yogyakarta Province. The

hypothesis was that some promising mutant lines could adapt well in such conditions so that they could be developed further in the region. It is our hope that drought tolerant sorghum can help local farmers overcome food and feed crisis during dry season and also as an alternative plant in producing biomass for supporting development of a sustainable agriculture. The scheme of combined tissue culture techniques and mutation breeding in sorghum was presented at the attached Figure.

4) Identification of DNA Markers in Rice

Blast disease caused by fungi *Pyricularia grisea* Cav. is one of the most important disease of rice. In the tropics, blast attacks are commonly found in both lowland and upland rice fields (Bonma and Mackill, 1998). The use of resistant variety has so far been the most effective way of controlling the disease. Unfortunately, the resistant variety generally cannot be long lasting because of out breaking the resistance by the new disease races (Kiyosawa, 1982).

Plant breeding for blast disease resistance has been aimed at using molecular markers for helping breeders incorporate complete and partial resistance genes into a new rice variety. A number of more than 30 resistance genes have so far been identified in rice (Kinoshita, 1998), and some of those genes have been mapped by using RFLP markers (Miyamoto, et. al., 1996; Monna, et. al., 1997; Wang, et. al., 1994; and Yu, et. al., 1996).

The local rice variety *Laka* was used as a resistance source in a plant breeding program for blast disease resistance. This variety is resistant to almost all races of *Pyricularia grisea* found in Indonesia. Meanwhile, *Kencana Bali* variety was used as susceptible control. This variety is susceptible to almost all disease races found in Indonesia. The contrast attributes between these two varieties are also found for their agronomic characteristics such as plant height, plant age, growth type, seed form etc. Inbred lines resulted from crossing between these two varieties will be of useful as permanent mapping population in identifying and analyzing genes controlling different characters found in the two varieties. RFLP analysis has been done for the inbred lines in order to search RFLP markers linked to blast-disease-resistance genes.

For identifying the combination of enzyme-markers showing polymorphism between *Laka* and *Kencana Bali* varieties, DNA was extracted from the two varieties by Dellaporta method (Dellaporta, et. Al., 1983). Another variety, *Asahan*, was also included in the identification. The extracted DNA was then digested with four restriction enzymes i.e. *Bam* HI, *Bgl*II, *Dra*I, and *Hind*III. The digested DNA samples were electophorized at 0.8 % agarose gel and transferred to nitrocellulose membranes by using alkalic solution 0.4 N NaOH. Afterwards, the transferred DNA were hybridized with 146 different RFLP markers from Rice Genome Research Program in Japan (Harushima et. al., 1998).

Hybridizing, labeling, and detecting the DNA band on X-ray film were done by using ECL kits from Amersham. The combination of enzyme-markers showing polymorphism was used for genotyping the inbred lines. RLFP procedure in

genotyping the inbred lines was the same as that in the identification of enzyme-marker combination showing polymorphism.

From that research work, some results have been obtained. A number of 144 inbred lines of rice have been resulted from crossing the *Laka* and *Kencana Bali* varieties. These inbred lines can be used for identifying DNA markers linked to the blast disease resistance, and also for being used in studying the inheritance of some important agronomic traits derived from the two varieties.

Based on the genotypic and phenotypic data, the DNA marker R2171 was linked to Chromosome 6, and the DNA marker R3089 to chromosome 7, with the resistance genes derived from *Laka* variety. Meanwhile, for detecting location of identified and other resistance genes, it is necessary to use additional markers and to test the inbred lines against blast disease with other races of *Pyricularia grisea*. This research is now still progressing under cooperation with the Plant Breeding Laboratory, Kyushu University, Japan.

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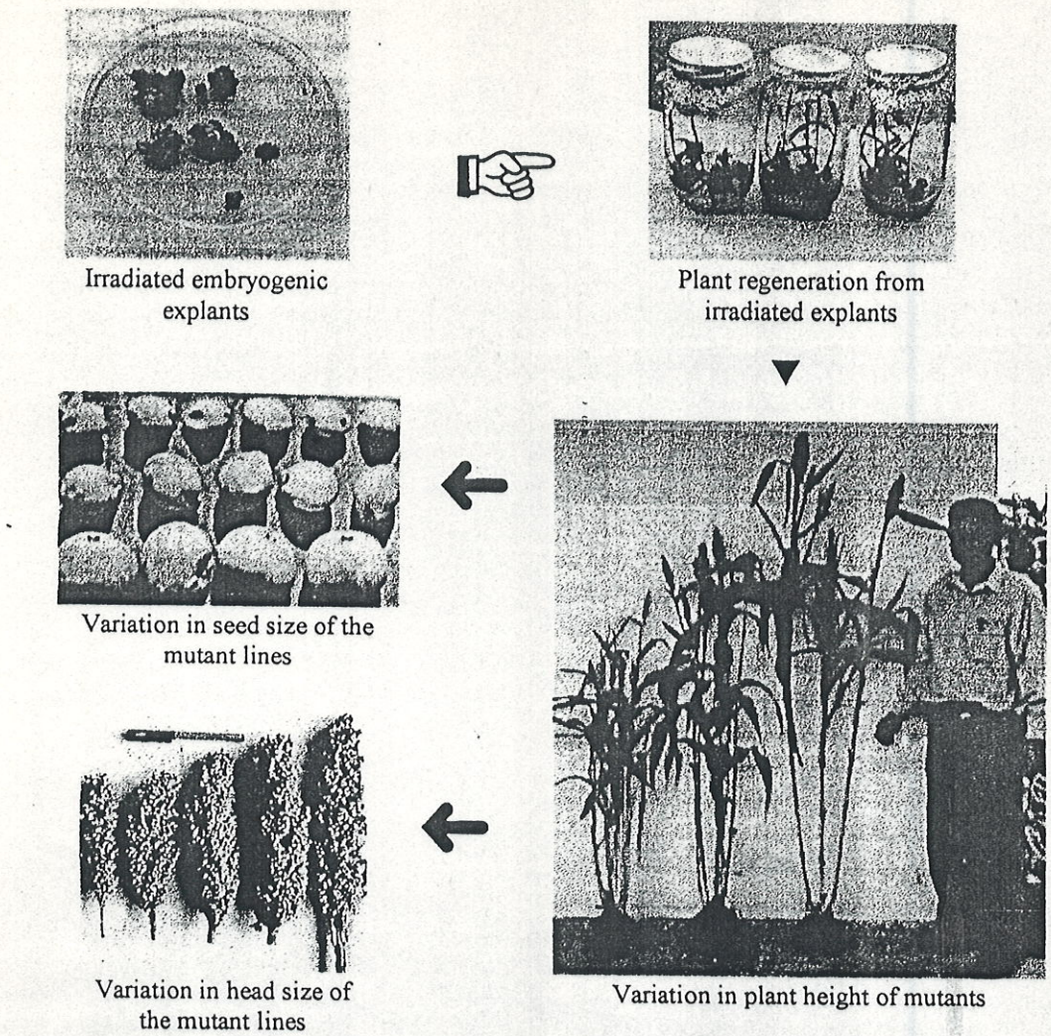

Figure 1. The scheme of combined tissue culture techniques and mutation breeding in sorghum.