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Accelerated purification of sorghum mutant line by using rapid cycling methods

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Abstract. Sorghum, a local food crop, can be developed on marginal land (drought, salinity, and acidity). Sorghum plant breeding takes a long time to get superior sorghum varieties. Rapid cycling technique is a method of accelerating breeding by repeating the life cycle of plants using immature embryo as planting material. The purpose of this research is to study rapid cycling techniques to accelerate the purification of sorghum mutant lines. In-vitro culture experiments were designed using a Completely Randomized Design (CRD) and Randomized Complete Group Design (RCBD) for in-vivo culture with six treatments of sorghum mutant lines with Super-1 variety as a control and three replications. The results showed that rapid cycling techniques effectively shortened the purification of sorghum mutant lines four weeks faster each generation than conventional methods. The height of GHP-1 mutant line (115.75 cm) is significantly shorter than the Super 1 variety, so it has the potential to be superior food sorghum and high production.

1. Introduction

Sorghum is a prospective plant to be developed in Indonesia. It has many benefits, wide adaptability, high productivity, requires relatively less input, resistant to pests and plant diseases, and is more tolerant of marginal conditions (drought, salinity, and acidity) [1]. The broad adaptability of sorghum makes it has an excellent opportunity developed in Indonesia in line with optimizing the used of marginal land [2].

Sorghum is not a native plant in Indonesia. The genetic diversity is still shallow, so plant breeding activities are needed [3]. Sorghum is better known as self-pollinating plants, but the potential for cross-pollination is quite significant. It reaches 30-60% when open panicle position. Because of the high potential for cross-pollination between sorghum plants, it is difficult to get pure mutants [4] so that it takes a long time to get the results of sorghum plant breeding. Sorghum plant breeding usually takes five months for each generation. It takes six generations to get pure sorghum mutants. However, recent advances in biotechnology and genomics have the potential to accelerate the purification of sorghum plants [5].

Rapid cycling technique is one method of accelerating sorghum breeding by repeating the life cycle of plants using the tissue culture method to shorten the lifespan of the sorghum harvest. The embryo rescue method can reduce the process for six weeks for each generation, which means to shorten the time of several months in sorghum breeding [4].



The rapid cycling procedure in wheat and barley have been published widely. This procedure can also be used in many wheat and sorghum genotypes. Ten wheat varieties and seven sorghum varieties from Kenya and Sudan have been propagated by the different treatment of pot size, day length, and watering intensity [6]. This research aims to get rapid cycling methods to accelerate the purification of sorghum mutant lines.

2. Material and Method

The research was carried out from February to June 2018 in the Tissue Culture Laboratory and the greenhouse of the Plant Breeding Group, Agricultural Division, Centre for Isotope and Radiation Application, National Nuclear Energy Agency.

The materials used in this study were MS (Murashige and Skoog) media, Sorghum embryos, tissues, spirits, alcohol, sterile water, and Clorox.

The tools used in this study were Laminar airflow cabinet (LAF), autoclaves, culture bottles, Petri dishes, hand-sprayers, tweezers, scalpels, Erlenmeyer, measuring cups, hotplates and magnetic stirrers, scales, push racks, pipettes, hoes, buckets, buckets, plant shears, calipers, rulers, cameras and stationery.

The genetic material used in this study was that some sorghum mutant lines resulted from the pedigree selection of Zh-30 irradiated mutant population by gamma at a dose of 300 Gy. The breeding of sorghum mutant lines has gone through a selection process and is in the M4 generation. The M5 generation obtained exciting characteristics compared to its controls. These sorghum mutant lines are GHP-1, GHP-2, GHP-3, GHP-4, GHP-8, and GHP-9 which have a short stem and good grain yield. In this study, there are two experiments. The first is in-vitro culture to grow the sorghum mutant immature embryo. The second one is in-vivo culture to obtain an immature embryo that would be used as explant for several repeated lifecycles of sorghum mutants in the purification process. In-vitro and in-vivo cultured experiments used Super-1 variety as national sorghum control.

2.1. In-vitro culture

Immature embryo sorghum mutants were grown on MS induction media, 3% sucrose, 0.2% Gelrite, Benzylaminopurin (BAP), Indole butyric acid (IBA) with a pH of 5.8. The embryos were sterilized using 70% ethanol for 5 minutes and also 1.05% Sodium Hypochlorite then rinsed with sterile aquades. Sorghum embryos were planted on MS media which had been supplemented with BAP and IBA hormones. Explants were cultured at 25C with a light intensity of 2100 lux for 16 hours per day. The explant was incubated in the culture room for a week. The acclimatization process was done when the roots, leaves, and stem explants grow perfectly. The experimental design used for in vivo culture is the Completely Randomized Design.

2.2. In-vivo culture

Acclimatized plants were planted in pots with a diameter of 30cm. Manure was given as 5 kg for 5 kg of soil. After seven days of age, plants were transferred in pots. Fertilizing was given twice when the plant is ten days after planting at a dose of 150 kg.ha-1 Urea, 200 kg.ha-1 SP36 and KCl 100 kg.ha-1 and second fertilization with a dose of Urea 150 kg.ha-1 at age 30 days after planting. All living plants are taken from each line to observe their agronomic characteristics. Observations were made on plant height (cm), the number of leaves, and stem diameter (cm) characters. The experimental design used for in vivo culture is the Randomized Complete Block Design.

2.3. Data analysis

There were ten replicates for each mutant in all experiments, which were laid out in a completely randomized design. Minitab software was used for statistical analysis (Minitab Inc.) The data were subjected to a one-way analysis of variance (ANOVA) followed by mean separation by the Least Significant Difference (LSD) test at $P < 0.05$.

3. Result and Discussion

An efficient and stable in vitro system is one of the ideal conditions that must be mastered when developing an efficient method of rapid cycling. The success of tissue culture in cereal plants is determined by several things, including genotype, type, age, explant condition, and regeneration media. Plant regeneration is genotypic specific so that the regeneration method in one genotype will be different from other genotypes [7], [8], [9].

Plant Selection for explants was made on the age selection of sorghum embryos. Age of plant will affect to sorghum embryos size as explant. Getting sorghum embryos less than ten days old are easily damaged and difficult to extract. According to Rizal et al. [4], the best collection of BTx623 sorghum line embryos is at up to 12 days after flowering (figure 1). Growing media in tissue culture also has a significant influence on the growth and development of explants. Explant growth response that is cultured depends on the interaction and balance between endogenous growth regulators that are in explants and exogenous growth regulators added to the media [10].

Benzyl Amino Purine (BAP) and Indole-3-butyric Acid (IBA) are the most widely used cytokinins and auxins in tissue culture. They are the most capable of stimulating shoot formation, inducing, and increasing root growth in various plants [11,12,13].

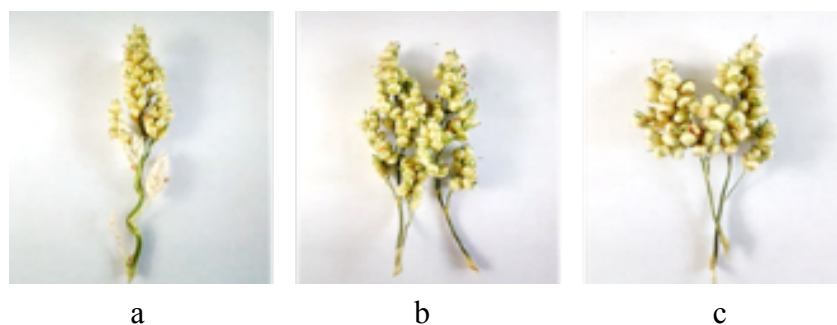


Figure 1. Sorghum panicle based on flower age; **a.** Panicle Sorghum 5 days after anthesis; **b.** Sorghum panicles 8 days after anthesis; **c.** Sorghum Panicles 12 Days after anthesis.

3.1. Induction of sorghum embryos

Three predominant obstacles for decades challenged sorghum tissue culture, namely toxic pigments (phenolics), low regeneration frequencies, and short duration of callus regeneration [14]. Here, we report a tissue culture system for sorghum, which has minimized these significant impediments. Using various plant growth regulators, such as Benzyl Amino Purine (BAP) indole-3-butyric acid (IBA) were evaluated to optimize the media.

Determinants in the growing media are the composition of inorganic salts, PGR, and the physical form of the culture media. Plant growth regulators are plant hormones that can stimulate the growth of individual cells or tissues of callus cells that have not been differentiated. Essential factors in the use of ZPT include; type, concentration, and sequence of use of PGR as well as the duration of plant induction on media containing PGR. There are kinds of ZPT, but the most commonly used are auxins and cytokines [15].

Table 1. Performance of plantlet height and root length some mutant sorghum in in-vitro culture

Genotypes	Plantlet height (cm)		Rooting (cm)	
Super 1	5,78	ab	4,52	a
GHP-1	5,7	ab	3,59	ab
GHP-2	6,29	a	2,88	ab
GHP-3	5,4	ab	3,39	ab
GHP-4	5	ab	4,23	a
GHP-8	4,7	b	2,01	b
GHP-9	4,75	b	3,55	ab

Note: The numbers followed by different letters in the same column are significantly different based on BNJ's further tests at the 5% level.

Observation of plantlet height at 14 days after embryo rescue (DAE) in Super 1 variety was not significantly different to four lines (GHP-1, GHP-2, GHP-3 & GHP-4) but substantially different to other lines (GHP-8 & GHP-9). Plantlet height differences between mutant lines and Super 1 variety can be caused by mutation effect influenced by gamma radiation. Higher radiation dose induced mutation massively. The more cells were damaged. It produced the raising of recessive genes such as dwarf planets that can appear [16]. IBA in tissue culture techniques plays a role in causing and increasing root growth in various jackfruit plants [11] and papaya [12]. IBA, in general, can spur growth better than NAA [17].

Root formation occurs because of the downward movement of carbohydrates, auxin, and rooting cofactor (substances that interact with auxin), which results in rooting [18]. Roots are very influential in vitro growth. The faster the plantlet forms roots, the quicker the process of metabolic balance in the plantlet's body. The primary function of roots is water and nutrient absorbent from the soil or the media to be supplied to plants when growing in vitro or the acclimatization process [17].

The sorghum plantlet in this study did not have shoots because of the use of hormones in its culture media. The cytokinin hormone used in low concentrations is only 0.5 ppm, while the auxin hormone used in higher levels is 2.5 ppm. It can maximize the growth of sorghum plantlet roots during the acclimatization process, and the plants can survive up to the field. [19], which states that the existence of primary roots will determine the level of success in acclimatization. The unsuccessful factor in preliminary research during the acclimatization process is due to the formation of plantlet roots is less than perfect. When planted in soil media, soil nutrient absorption is less than the maximum, which causes the plants not to develop properly.

**Figure 2.** Sorghum Plantlet at 14 DAE Resulted from Tissue Culture with Rapid Cycling Techniques.

3.2. *In-vivo culture*

3.2.1. Plant height. Observation of plant height at week after planting (WAP) in Super Varieties 1 (197.89 cm) was significantly different from other lines. Super 1 variety is the highest plant in this study; when compared to the description in Super-1 variety, there is a difference in plant height that is 7 cm different. The shortest plant in the study was GHP-1 (115.75 cm), the height less than 120 cm so that it is easy to harvest and resistant to lodging (Table 2).

The primary purpose of breeding sorghum mutant lines is to change phenotypes and genotypes for the better. The diversity that arises from each genotype resulting from irradiation in each different character is due to differences in the genetic constitution and the response to gamma-ray radiation. Mutation induction can cause diversity in M2 generation populations due to the high segregation of mutated genes [20,21]. Mutations with gamma-ray irradiation increase production, early maturity, pest resistance, pathogens, lodging, and better seed quality under optimal environmental conditions. It can change the morphological and agronomic characteristics of plants, such as reducing plant size, accelerating harvest, changing colors, and fruit skin [15].

3.2.2. Stem diameter. Observation of 10 WAP on stem diameter of Super 1 variety was significantly different from GHP-2, GHP-3, GHP-8, and GHP-9 but not substantially different from GHP-1 and GHP-4. GHP-9 (2.13 cm) showed the largest diameter and plants with the smallest diameter produced by Super-1 Variety (1.20 cm). Ningrum [22] states that large stems are directly proportional to large panicle stems because they function as a panicle buffer and minimize lodging risk. Besides large stems have more vascular bundles that help strengthen the plant's upright.

Table 2. Data on Plant Height, Stem Diameter and Number of Leaves of Sorghum Mutant Lines in Greenhouses Age 10 WAP Embryonic Tissue Culture Results in Rapid Cycling Techniques.

Genotype	Plant height (cm)		Stem diameter (cm)		Number of leaves	
Super 1	197,89	a	1,20	c	9,56	ab
GHP-1	115,75	c	1,65	abc	10,5	a
GHP-2	120,71	bc	1,80	ab	9,14	ab
GHP-3	132,00	bc	1,80	ab	10,2	a
GHP-4	145,00	b	1,45	bc	7,17	c
GHP-8	144,00	bc	1,78	ab	8,50	bc
GHP-9	143,00	bc	2,13	a	10,25	a

Note: The numbers followed by the same letters in the same column are not significantly different based on BNJ's further tests at the 5% level.

3.2.3. The number of leaves. Observation of the number of leaves aged 10 WAP in Super 1 variety was significantly different from GHP-4 and GHP-8 but not substantially different from other lines. The highest number of leaves is produced by GHP-1 (10.50 strands) and lines with the least average amount of leaves produced by GHP-4 (7.17 strands). Observation of the number of leaves is needed to be an indicator of growth as supporting data to explain the growth process that occurs as in the formation of plant biomass. At the beginning of the growth of the leaf, plants have not been actively photosynthetic. New leaves are photosynthetically active at a later stage of development and have an essential role in the growth process as long as the roots have not yet appeared. Leaves replace the role of roots in absorbing minerals needed for the growth process [17]. [23] reported that the number of leaves of

sorghum plants correlated with the length of the vegetative period, as evidenced by each addition of one leaf requiring 3-4 days.

3.3. Rapid cycling in sorghum plants

Rapid generation-advancement techniques or commonly called rapid cycling have been developed to accelerate plant breeding and support breeding in many plants, for example in tomatoes, soybeans, wheat, chickpea, rice and cotton [4,24,25,26,27,28,29]. The fundamental component of plant breeding creates new varieties. The time needed to get a new line is often a significant time constraint. The methods commonly used to accelerate plant breeding are single seed descent (SSD) and shuttle breeding. SSD is an old technique first proposed by [30], then modified by [31]. In this method, only one seed from the F₂ plant population is planted into F₃. This process is repeated in the next generation up to F₅/F₆ as the plant approaches a high degree of homozygosity. Only one seed is needed per plant in the early generation. It can be planted in small pots or tubs with limited watering and high light exposure in small areas like in the greenhouse [32].

The advantages of the single seed descent method were easy to handle segregated populations, require less land, shorter time in forming lines, suitable for greenhouses and off-season nurseries. There is no natural selection of the population, and each line originates from F₂ plants different, higher diversity. The disadvantage of the single seed descent method was genetic loss might occur if genetic seed germination is low. The number of F₂ seeds planted must be calculated appropriately, and it takes more time to harvest than the bulk method [33].

After the flowering phase, the embryo is cut and cultured, thus avoiding mature seeds and avoiding dormancy in the seeds, thus saving several weeks. The rapid cycling procedure has recently been applied to sorghum. It up to six generations produced in one year using small pots, continuous lighting, limited watering, and embryo rescue [34]. The *in vitro* stage is also ideal for taking tissue samples, for example, leaf samples for DNA extraction [35]. GHP-1 line is the sorghum promising mutant line observed in the rapid cycling method in this study. The results of the research [4] using the embryo rescue method can shorten Sorghum breeding time.

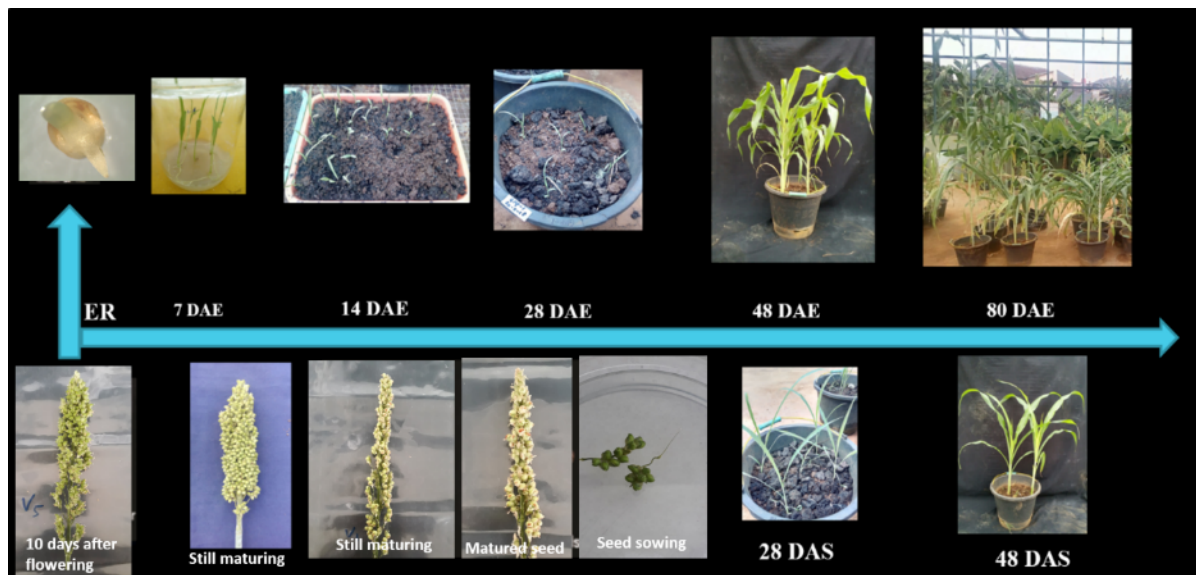


Figure 3. Rapid Cycling Method in Sorghum Plants

GHP-1 takes six weeks to mature seeds, harvest, and post-harvest before the seeds are ready to be planted again. The embryo rescue method can shorten the process for six weeks for each generation, which means to reduce the time for a few months in sorghum breeding. It can Accelerate the breeding

of sorghum plants resulting from crossing. Panicles are harvested at the age of 10-12 days after flowering for embryo rescue procedures. It planted on Murashige and Skoog (MS) media for one week. After one week it is planted again in Yoshida's solution for one week. Furthermore, it is planted in the ground using a diameter of 25cm pot. Seed in a panicle is ready to be harvested, seeding through embryo culture is prepared more quickly four weeks than ordinary panicle seeds.

In this study, sorghum embryos were taken ten days after flowering. Furthermore, it is planted on culture media for one week, then planted on acclimation media for two weeks. After two weeks it is planted in soil media in a 30cm diameter pot. The vegetative phase takes 68 days, and the generative phase takes 55 days. The total time to plant an embryo until the average harvest is 123 days. A comparison with sorghum plants grown in the field only takes 122 days. The vegetative phase takes 67 days, and the generative phase takes 55 days.

Table 3. Calculation of Harvest Time in Rapid Cycling and Conventional Methods

Phase	Methods	Cycle 1 (days)	Cycle 2 (days)	Cycle 3 (days)	Difference
vegetative	<i>rapid cycling</i>	68	68	68	3 days late
	conventional	67	67	67	
generative	<i>rapid cycling</i>	25	25	25	90 days earlier
	conventional	55	55	55	

The acceleration of sorghum breeding is done by shortening the generative phase. In the rapid cycling method, the generative phase can be shortened to 20 days because the embryo is harvested at the age of 10 days after complete flowering. It speeds up 30 days to wait for physiological maturity on sorghum plants.

In this study, the vegetative phase of plants with the rapid cycling method is one day slower than the conventional way. Sorghum plants are C4 plants and need sufficient sunlight to maximize their photosynthesis process [36]. In the traditional method, sorghum is grown in the field with an average light intensity of 45.900 lux. Sorghum in the rapid cycling method was planted in a greenhouse with a light intensity of only 6.630 lux. This difference in light intensity causes the vegetative phase of sorghum plants to be longer.

The use of the rapid cycling method was effective compared to conventional methods. The generative phase was reduced so that it can speed up the purification time because of different harvest time of 30 days. The accumulation of time that can be shortened if planting five generations is carried out in the purification of sorghum lines is 25 weeks faster than conventional methods. It is consistent with the results of research [4] using the embryo rescue method that can shorten Sorghum breeding time. It usually takes six weeks for seed maturation, harvesting and post-harvesting before the seeds are ready to be planted again.

4. Conclusion

- The use of BAP and IBA media effectively induces sorghum embryos to become perfect plantlets within 14 days can be acclimatized so that they can support rapid cycling techniques effectively to accelerate the purification of sorghum mutant lines four weeks faster than conventional methods.
- GHP-1 is very potential to be superior food sorghum because of its short plant height (≤ 120 cm) and excellent production.

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