

The Genetic and Morphoagronomy Character Diversity of Black Soybean Plant (*Glycine soja* (L.)): Responses to Mutation Induction by Gamma Rays Irradiation and Ethyl Methane Sulfonate

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Abstract

Indonesia has a high dependence on imports of black soybeans. National black soybean needs continue to increase. Therefore, some efforts are needed to increase soybean production. Genetic resources of Black Soybeans with high diversity is also needed to develop superior varieties. One of the ways to increase soybean production is by mutation induction. Therefore, genetic resources of Black Soybeans with high diversity is needed to develop superior varieties. Mutagenesis using gamma rays and EMS can be used to increase genetic diversity. Doses used in this research were 1000 Gy gamma rays, 1% EMS, and combinations. This research was using a completely randomized design with morphoagronomy and molecular parameters. Morphoagronomy parameters observed were plant height, leaf area, seed weight, number of leaves, number of pods, and number of branches. ISSR markers with four primers (UBC 888, ISSR3, UBC876, and UBC889) were used to determine genetic variation of Black Soybeans. Based on the analysis of variance (ANOVA), it was shown that all parameters were able to give an effect on the morphoagronomy of black soybean plants. Gamma rays 1000 Gy were more effective in improving morphoagronomy of the plant in terms of plant height, seed weight, number of leaves, number of pods, leaf area, and number of branches. UBC 888 was the most effective primer to identify the genetic diversity of black soybeans that have been given mutation treatments.

Keywords: Black soybean, EMS, gamma rays, genetic variation, morphoagronomy.

INTRODUCTION

Black soybean (*Glycine soja*) is one of the members of the Leguminosae family [1]. The Ministry of Agriculture and related institutions in Indonesia have released several superior soybean varieties such as Detam, Cikurai, Malika, Mutiara, etc. This research was using Detam 3 variety because it was one of the superior types of a black soybean variety that is still rarely studied. Detam variety has *anthocyanin* levels of 222.49 mg.100g⁻¹, and *isoflavone* levels (genistein 0.65 mg.g⁻¹ and daidzein 0.5 mg.g⁻¹) [2]. The lack of studies on this variety provides an opportunity for researchers to explore many unknown characters. Besides, Indonesia has a high dependence on imported beans, especially soybeans. In fact, Indonesia has a huge opportunity to increase legume production [3]. Based on data from the Central Statistics Agency (BPS), domestic soybean production was only 982.598 tons. Meanwhile, to meet the domestic needs, Indonesia has to import 2.6 million tons of black soybeans.

The main use of black soybean is for the raw material of soy sauce. At present, black soybeans

are starting to be considered as raw materials for processing soybeans such as tempe and tofu because of their good content for people with diabetes mellitus [4]. Black soybeans contain *anthocyanins*, *isoflavones*, and *saponins* [5]. Those are antioxidants that play an important role in the regeneration of damaged cells. The more widespread use of black soybean has shown that it is necessary to increase production to meet domestic needs.

Due to low soy production at this time, it is needed to assemble superior varieties of soybeans. The availability of high diversity genetic resources is needed for this purpose. Mutagenesis is one of the ways to expand the genetic diversity of soybeans. Mutagenesis can be done using chemical and physical mutagens. One of the chemical compounds commonly used for mutagenesis is Ethyl Methane Sulfonate (EMS), while gamma rays are often used for physical mutations [6].

Research by Hanafiah on mutations in soybean varieties Agromulyo using 200 Gy treatment gamma-ray irradiation produced the highest diversity plants [7]. Gamma rays irradiation with the strength of 250 Gy was also gave an effect of increasing growth in almost all parameters of observation (shoot length, roots, fresh weight and leaf dry weight) to the *Canscora decurrens* plant [8].

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EMS causes mutations at the DNA level by changing DNA bases (causing point mutations). It made slight damage to chromosomes, so it is beneficial in plant breeding activities [9]. Point mutations are also passed down to the next generation [10]. Whereas, soybean seeds soaked in 20 mM EMS for 10 hours produced soybean mutants with a total number of pods, contents, pod weight before drying, pod weight after drying, number of seeds, the weight of planting seeds, and weight of 100 seeds [11]. EMS mutagens can also be used at 0.05% to 2.5% concentration with 3 to 24 hours of soaking time [12,13].

Evaluation and characterization of mutant crops requires a long time and generally limited to morphological, anatomical, and biochemical characters. Therefore, it needs to do an efficient method that is not dependent on plant development [14]. Molecular characterization can be used to accelerate the process of crop selection. One of molecular the markers commonly used to detect polymorphism is Inter Simple Sequence Repeat (ISSR). ISSR is an effective technique in the genetic analysis of plant breeding programs. ISSR marker provides a quick method, low cost, and requirement for only small quantities of DNA [15], no need for DNA sequence information before amplification, high stability, and abundance of genomic information [16]. The percentage of Soybean's DNA polymorphism by using ISSR marker is about 70-100% [17].

Combination of gamma-ray irradiation and EMS as mutagen is still rarely applied to soybean plants. Previous research showed that EMS and gamma rays can increase the frequency of mutations Chlorophyll and sterile pollen in soybean MAUS-71 and JS-335 variety. [18]. Based on these facts, it was needed to observe the morphoagronomy character and genetic diversity in black soybeans induced by gamma-ray irradiation and EMS by using ISSR.

MATERIAL AND METHOD

Seeds of black soybean (Detam 3) were obtained from Research Institute for Various Nuts and Tubers (Balai Penelitian Tanaman Aneka Kacang dan Umbi - BALITKABI). This research was using a Completely Randomized Design (CRD).

Induction of Mutation by EMS

EMS 1% was dissolved it in Sodium phosphate buffer solution (pH 7) and DMSO 4%. The

treatment was carried out at room temperature [19].

Induction of Mutation by Gamma Rays Irradiation

Black Soybeans were irradiated by 1000 Gy dose of gamma rays. We used CO-60 Gammacell 220 irradiator (dose 4585.5 Gy.hour⁻¹) at the National Nuclear Energy Agency of Indonesia (BATAN).

Table 1. Treatment Detail

Treatment Code	Treatments	Number of seeds
Control		
T0-without mutation	Seeds soaked in phosphate buffer pH 7 for 6 hours.	15
T1-without mutation	Seeds were planted without soaking.	15
Gamma rays irradiation		
T2-1000Gy	Seeds were irradiated by 1000 Gy of gamma rays.	100
EMS		
T3-1%	The seeds are soaked with pH 7 phosphate buffer for 6 hours, then soaked in EMS 1% for 6 hours.	100
Combination		
T4-1000Gy+1%	The seeds were irradiated with 1000 Gy gamma rays, then soaked in pH 7 phosphate buffer for 6 hours, and soaked in EMS 1% for 6 hours.	100

Planting Induced Mutations Seeds

The planting media used in this research was 3 kg of soil and sand with 1:1:1 of compost addition to each polybag. A total of 3 kg media was calculated from the weight of tillage per one hectare that is 2 million kg, and the optimum population of soybean per hectare that is 255.000 plants [20,21]. The media watered when the field capacity was 100% before use. A soybean seed planted in each polybag at ± 3 cm depth and carried out simultaneously until the harvest time.

Observation of Genetic Diversity

DNA Isolation, Purification and Quantification

The third or fourth apical leaf of black soybean was used for DNA isolation by using Doyle and Doyle protocol [22] with modification. DNA purity and concentration were determined using nanodrop (Nano 200-1002).

Electrophoresis

Electrophoresis was running at constant voltage 60 V for 60 minutes in 1x TBE buffer pH 8.0. The agarose gel used for DNA genome was 1% and 1.5% for PCR product. DNA ladder

(Promega) 100-1000 bp were used as the molecular size standards.

DNA Amplification by Using ISSR Marker

The components of the PCR reaction mixture and ISSR primers were used for this study can be seen in Table 2. The PCR reaction was performed using BioRad Thermal Cycler (Fig. 1).

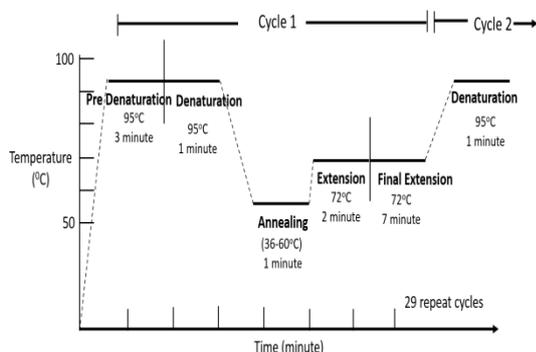


Figure 1. The PCR reaction

Morphoagronomy Character Observation

The morphoagronomy character observed were plant height (cm), seed weight (g), number of branches, number of pods, number of leaves, and leaf area. These characters were observed 30 days after harvest time.

Data Analysis

The data of morphoagronomy character of black soybeans were analyzed by using one way ANOVA then followed by DMRT test at 5% level to find out the best treatment of mutation induction. The ISSR product was scored for each primer then followed by the calculated percentage of polymorphism band. Statistical analysis was performed using the Paleontological Statistics Software Package for Education and Data Analysis (PAST). Dendogram was made via Un-weighted Pair Group Method with Arithmetic Average (UPGMA).

RESULT AND DISCUSSION

DNA Amplification

The result of DNA amplification using ISSR markers (Fig. 2) informs that there was a new DNA band appeared in the mutants, which where

missing in the control. The different sizes of the DNA band indicated a change in the nitrogenous base as the result of mutation. When there is a mutation of DNA, the primer that initially attached to this site could not recognize it. The primer either did not attached to the initial site or attached to the new site formed by mutation. The amplification of this new site resulted in a new band with different sizes and thicknesses, so it made a polymorphism.

There were 45 fragments amplified from four primers. The number of fragments produced from each primer varies between 9-14 fragments. The result of polymorphic percentage ranged from 58.33-92.86%. ISSR3 produced the lowest polymorphic percentage (58.33%), and UBC888 has the highest value (92.86%) (Table 2).

The highest value of PIC, EMR and MI were generated by UBC888, the lowest value of PIC and MI were indicated by ISSR3, and the lowest values of EMR and RP were resulted by UBC889 (Table 2). PIC Values for dominant markers were between 0.0-0.5. The higher the PIC value, the better was the primer in determining genetic variation [22]. The EMR value is used to determine the number of polymorphic fragments in the observed sample. The higher EMR value showed the more effective primer in produce polymorphic fragments [23]. The MI value is used to determine the primer index in producing polymorphic fragments [24]. The RP value is used to determine the strength of a primer in producing a clear fragment. The higher the value of Rp, the better a primer in producing clear fragments [25].

Phylogenetic analysis

The similarity between Detam 3 and three other mutant varieties was based on the result of primer amplification. Phylogenetic analysis between accessions using PAST software were presented in a dendogram expressed by similarity value (Fig. 3). This result was calculated from three replication of each treatment except the control.

Tabel 2. ISSR primer amplification, % polymorphism of mutant soybean through gamma rays and EMS induction

No	primer	Ta (°C)	TNB	NPB	PB(%)	PIC	EMR	MI	RP
1	UBC889 5''-DBDACACACACACAC-3''	53.0	9	6	66.67	0.45	4	1.80	11.82
2	ISSR3 5''-GTGTGTGTGTGTGTAC-3''	50.0	12	7	58.33	0.41	4.08	1.67	17.09
3	UBC876 5''-GATAGATAGACAGACA-3''	52.0	10	8	80	0.43	6.4	2.73	13.82
4	UBC888 5''-BDBCACACACACACA-3''	53.0	14	13	92.86	0.49	12.07	5.93	15.82

Notes: TNB: Total Number Band, NPB: Number Polimorphic Band, PB: Polimorphic Band, PIC: Polymorphic Information Content, EMC: Effective Multiple Ratio, MI: Marker Index, RP: Resolution Power

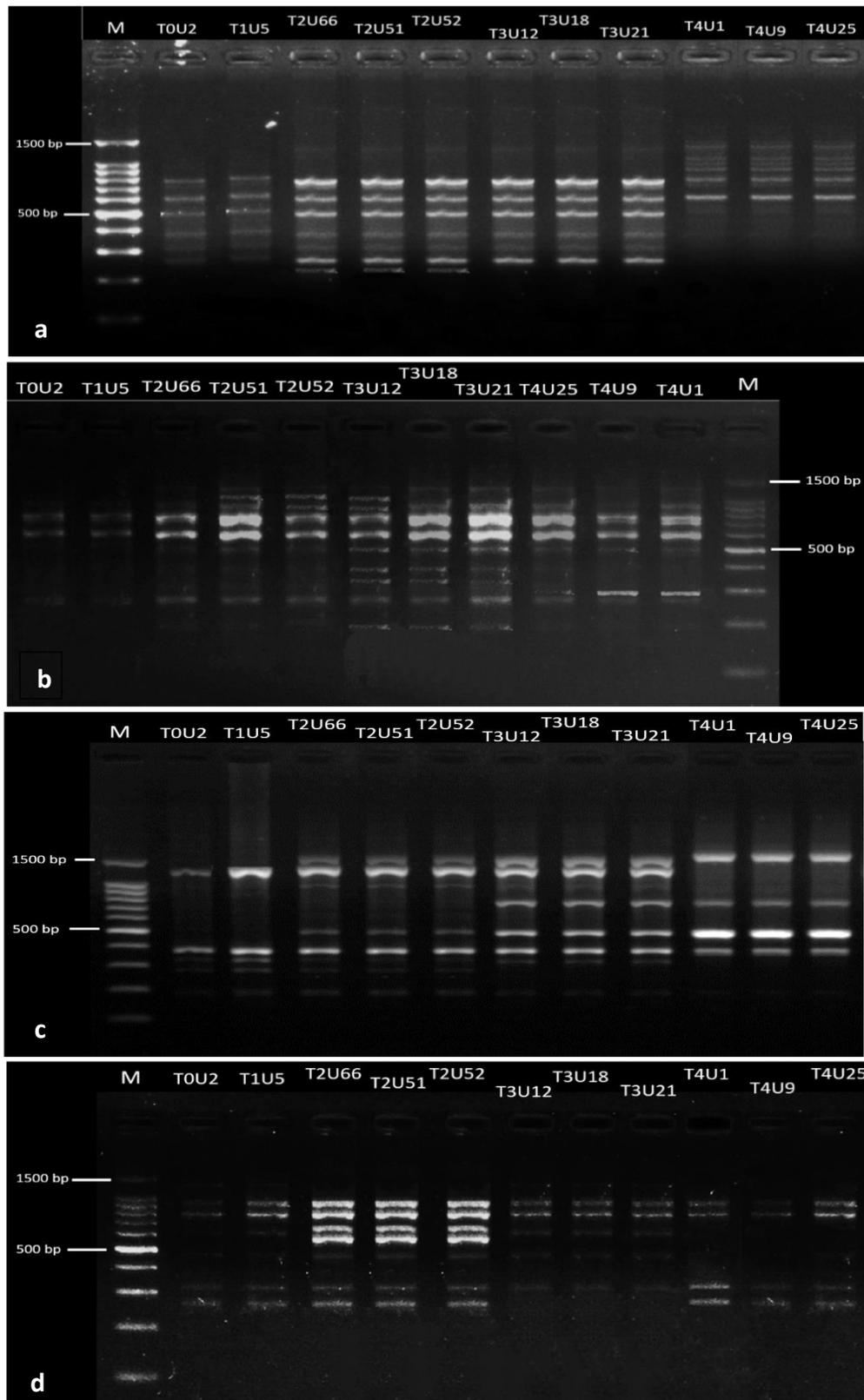


Figure 2. Profile of DNA amplification band for each primer (a) UBC 888, (b) ISSR3, (c) UBC876, (d) UBC889.

Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

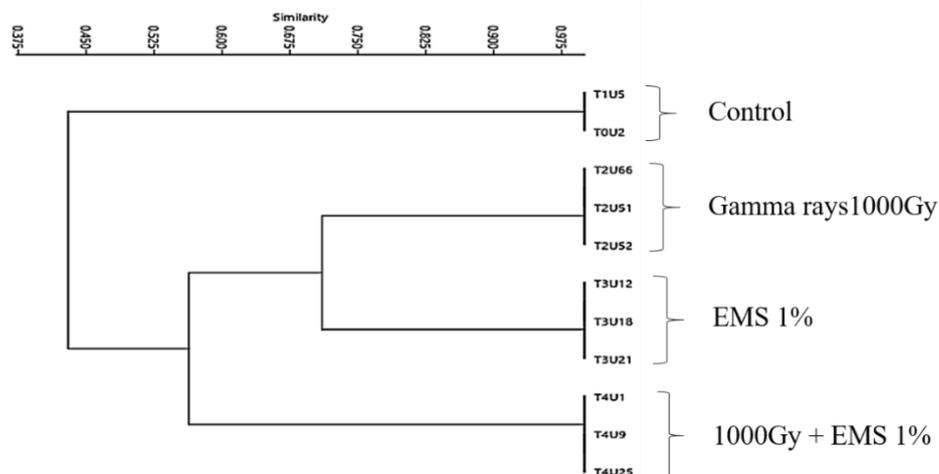


Figure 3. A dendrogram of black soybeans (Detam 3) mutated by gamma rays and EMS

Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

The similarity of each treatment can be determined by the genetic coefficients. The genetic coefficient value was about 0 to 1. The closer value to 1 means the more similar genetically, and vice versa [26].

The dendrogram in Figure 3 showed that between the control and others, there was a 0.425 similarity value. The sample of combination treatment has 0.55 in similarity with EMS and Gamma rays irradiation. And between the sample of gamma rays and EMS treatment has 0.69 similarity value. It showed that both EMS and gamma rays irradiation were effective methods to increase the genetic diversity of Black Soybeans.

Analysis of mutation treatment by EMS and Gamma rays irradiation showed changes in each treatment. It was caused by the change of DNA sequences so that the primer attachment site changed too. The research by Selvarasu showed

that ISSR markers were able to show polymorphisms in *Gloria superba* plants induced by mutagens [27]. Aswandi also carried out research on the *Solanum lycopersicum* plant and reported that ISSR was a reliable primer for the initial and screening of mutagen [28].

Analysis of the effect of gamma ray irradiation and EMS on black soybean morphoagronomy

Morphoagronomy parameters observed in this study were plant height, seed weight, number of leaves, number of pods, and number of branches. Based on the analysis of variance (ANOVA), it was shown that all parameters observed had an $F_{count} > F_{table}$ of 5% level, which means that there was an effect of gamma-ray and EMS treatment on the morphoagronomy of black soybean plants. The significantly different results were further tested by Duncan Multiple Range Test (DMRT) on a 5% level (Table 3).



Figure 4. Habitus of Mutant Black Soybean Plants

Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

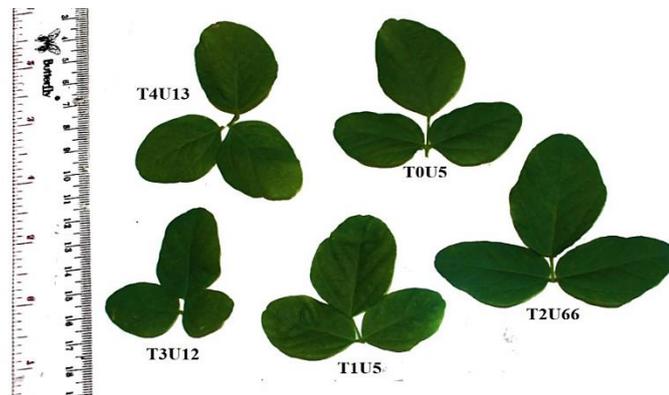


Figure 5. Leaf Area of Mutant Black Soybean Plants

Description: *T= treatment, U=replication. T0= planted without mutation (Seeds soaked in phosphate buffer pH 7 for 6 hours), T1= planted without mutation (Seeds were planted without soaking), T2= 1000Gy, T3= 1% EMS, T4= 1000Gy+1% EMS.

Table 3. Gamma rays irradiation and EMS effect to morphoagronomic character

Treatments	Plant height (cm)	Seed weight (g)	Number of pods	Number of leaves	Number of branches	Leaf Area (cm ²)
Gamma ray 1000Gy	46.71 ^c	3.85 ^c	24.87 ^c	78.9000 ^d	3.44 ^b	16.17 ^d
EMS 1%	24.69 ^a	1.77 ^a	13.06 ^a	36.1923 ^a	2.33 ^a	10.54 ^a
Gamma ray + EMS	54.46 ^d	3.30 ^b	22.24 ^b	67.3600 ^b	2.40 ^a	14.11 ^b
Control (with buffer soaked)	41.79 ^b	3.03 ^b	20.53 ^b	74.0000 ^c	3.47 ^b	15.15 ^c
control (without buffer soaked)	41.70 ^b	3.00 ^b	20.53 ^b	74.2000 ^c	3.53 ^b	15.00 ^c

*numbers were followed by the same notation of DMRT (5%) means not significantly different

The plant height, leaf area, and the number of leaves of black soybean plants treated with gamma-ray irradiation and EMS were varied. The seed weight and the number of pods indicate that the treatment combination had the same effects as the control treatment. The number of branches in black soybeans treated with EMS and combination treatment tends to be the same. Table 3 showed that the EMS treatment caused a decrease in all quantitative variables. This result was in line with Wahyudhi, who reported that EMS could cause a decrease in the growth of Gogo rice seedlings [29].

Based on the result of this study, gamma rays tend to have a positive effect compared to other treatments. It has the same result with Meliala's research that gamma-ray treatment increased phenotypic diversity and plant height in upland rice [30]. Ragapadmi also reported that treatment with EMS has the ability to damage cells more than gamma-ray irradiation [31]. According to Astuti, gamma rays irradiation at certain doses can stimulate plant growth [32].

The growth was due to loss of meristem cell ability in deviced so that the cell activity was increased.

Gamma rays are short electronic waves with a high-energy level that can bind into atoms or molecules to produce free radicals in plant cells. Mutation induction using gamma rays irradiation produced the most mutants (around 75%) compared to other treatments such as chemical mutagens [33]. The diversity caused by mutagens was different in each species and even varieties. It can have both positive and negative effects depending on the concentration and duration of immersion given due to differences in genetic material and tolerance in responding to mutagen.

CONCLUSION

Gamma rays and EMS were able to produce genetic diversity in black soybeans Detam 3 varieties. UBC 888 was the most effective primer to identify the genetic diversity of black soybeans that have been given mutation treatments.

Gamma rays 1000 Gy were more effective in improving morphoagronomy.

The limitation of this research was the lack of mutagen dosage variation. For future study, it needs to use more doses of gamma rays and EMS. It is also a need to use more primers and specific genes to determine gene to determine the genetic diversity in mutated plants.

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