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IMPROVING HEAT TOLERANCE IN POTATO (*SOLANUM TUBERSOUM* L.) CULTIVAR BIO GRANOLA THROUGH GAMMA RAY IRRADIATION AND *IN VITRO* SELECTION

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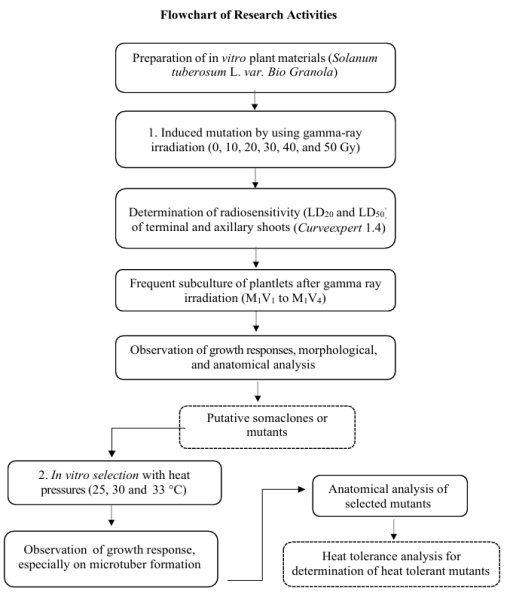
Graphical abstract



Abstract

The potato variety Bio Granola is resistant to late blight disease, however it can only adapt to highland areas. Sexual hybridization in potatoes is constrained by biological barriers which make mutagenesis is considered to be applied. This study aimed to determine the radiosensitivity response of *in vitro* shoots to gamma ray irradiation and to analyze the morphological and anatomical characteristics after *in vitro* selection under high temperature pressures. This study consisted of two experiments: (1) Induced mutation by gamma ray irradiation (0, 10, 20, 30, 40, and 50 Gy) using explant types of terminal and axillary shoots from less and more than 1 year period of motherstock, and (2) *In vitro* selection with temperature pressure (25, 30, and 33 °C). Observations were conducted on growth responses, morphological, and anatomical characters. The results showed variations in growth response and morphological characters of plantlets after gamma ray irradiation. Anatomical analysis showed changes in stomatal density and size, and number of chloroplasts. Gamma ray irradiation combined with *in vitro* selection under temperature pressure successfully generated putative mutants derived from potato cultivar Bio Granola with enhanced tolerance to high temperatures. The most effective method utilized terminal shoot explants from young motherstock (<one year old) treated with irradiation doses of 20–30 Gy. This treatment promoted high survival rates, vigorous growth, and the capacity to form microtubers under 30 °C stress. Plantlets that produce microtubers under heat stress are considered as potential heat-tolerant genotypes. Further evaluation through field trials in medium- and low-altitude environments is required.

Keywords: Anatomical characters, growth response, heat stress, microtuber, radiosensitivity

1.0 INTRODUCTION

Potato varieties (*Solanum tuberosum* L.) have been cultivated in Indonesia since the early 19th century in highland areas. Studies showed that potato crop research focuses on the comparison of agronomic properties [1], cultivation methods [2], physiological, biochemical, and molecular [3] aspects of the growth and production of potato crops. Most of the consumption of potatoes by the Indonesian people tends to continue to increase, especially in the form of processed potatoes such as French fries and chips. Potato production in Indonesia itself is still dominated by Granola varieties, which reach more than 80% of the planting area and are spread across potato planting areas in the highlands [4].

Bio Granola is the result of crossing Granola variety with Genetically Modified Organism (GMO) Katahdin SP951, which has the advantage of being resistant to late blight (*Phytophthora infestans* L.) [5]. Geographically, Bio Granola adapts to low-temperature highland environments, Therefore, genetic modification is necessary to increase its tolerance to adapt to lowland areas.–One approach that can be done is to induce mutations combined with *in vitro* selection techniques.

Induced mutation using gamma rays is commonly used in plant breeding [6]. Gamma-rays are ionizing radiation [7], which interacts with atoms or molecules and produces free radicals in cells that induce physiological, biochemical, cytological, genetic, and morphological changes in plant cells and tissues [8, 9]. Induced mutation may rise to genetic diversity by utilizing *in vitro* culture techniques, and the selection process is carried out in conjunction with the physiological activity of the plant [10, 11].

High temperature stress is an essential factor influenced by the environment. It may cause various physiological and biochemical changes in plants, including cell membrane damage, protein denaturation, and accumulation of reactive oxygen species [12, 13]. These changes can interfere with metabolism and photosynthesis, ultimately reducing yields and quality in plants that are intolerant to high temperatures [14].

Mutation breeding is extensively utilized in vegetatively propagated crops, as it rapidly generates genetic variation without the need for sexual hybridization [6, 7]. In potatoes, conventional hybridization is particularly difficult due to differences in chromosome numbers, sterility, sexual incompatibility, and the fact that several cultivars do not produce flowers [15]. *In vitro* selection is an effective complement to mutagenesis, as it enables the screening of plantlets under controlled stress conditions and facilitates the early identification of putative mutants prior to field evaluation [3, 10]. The combined treatment of gamma ray mutagenesis and *in vitro* selection has been reported in several crops such as rice, stevia, and tomato to enhance tolerance to abiotic stresses, underscoring its potential for developing heat-tolerant potato varieties [6, 17, 24]. This study aimed to determine the radiosensitivity response of *in vitro* shoots of Bio Granola to gamma ray irradiation and to analyze the morphological and anatomical characteristics after *in vitro* selection under high temperature pressures.

2.0 METHODOLOGY

The genetic material used was Bio Granola variety. The experimental design was a Completely Randomized Factorial Design (CRFD) with three factors, namely (1) dose (D) which consisted of 6 levels (0, 10, 20, 30, 40, and 50 Gy); (2) explant source or motherstock (S) consisting of 2 levels, namely D48B (young, less than 1 year *in vitro* period) and D48L (old, more than 1 year *in vitro* period); and (3) explant type (E), which consist of 2 levels, namely terminal shoots (T) and axillary shoots (A). Each treatment was repeated 4 times, and each repeat consisted of 10 explants.

Explants were isolated from the plantlets then planted on a 1/2K0.5 regeneration medium, which was MS media (Murashige and Skoog, 1962) diluted twice and supplemented with 0.5 mg L^{-1} kinetin. Initial explants (M_0V_0) were incubated for one week in culture room with light intensity of 1000−1500 lux, photoperiodicity of 16 hours/day, and a temperature of 25 °C. The explants were irradiated with gamma rays, and subsequently sub-cultured on 1/2K0.5 media then incubated in room culture under the same conditions as the previous stage for 3-4 weeks. Subculture was carried out four times until a putative mutant population resulting from the fourth subculture (M_1V_4) was produced.

Each individual plantlet that grows was assigned by a number or code then visually observed for its phenotype. If there was a visual difference, a letter code was added after the numerical code until the mutants were assumed to be uniform (solid).

The M_1V_4 materials were multiplied until M_1V_5 and M_1V_6. They were planted on microtuber induction media (MS media supplemented with paclobutrazol 2 mgL^{-1} and sugar 150 gL^{-1}), and were challenged by heat pressures in incubator with normal temperature (25 °C) as the control, 30 and 33 °C. Temperatures of 30 °C and 33 °C were selected as screening pressures because they reflect the average daytime conditions in Indonesia's midland and lowland regions, where heat stress often leads to plant mortality or reduced tuber formation. Consistent with this approach, Gautam *et al*. (2021) applied 30 °C, while Guillemette *et al*. (2024) used 33 °C as selection pressures. The explants were incubated in a dark condition for 4 weeks after culture (WAC). After *in vitro* selection, explants were transferred into the culture room for recovery. The growth of the explants was then observed until microtubers were formed. Observations were conducted at 4 WAC in the M_1V_1 population after gamma ray irradiation and M_1V_4 after heat pressure. Variables observed in the M_1V_1 population

including survival rate such as percentage of live explants (%), lethal dose 20% (LD_{20}) and 50% (LD_{50}), as well as morphological characters (visual) including roots (non-hair roots, hair roots), root length (long, short), stem color (green, yellow, brown), leaf color (green and reddish green), bud stem color (green, reddish green, yellow), and leaf arrangement (wide, bud).

For M_1V_4 plantlets incubated in the room culture (control) and incubator (heat pressure) were observed for their growth responses and anatomical characteristics. The observed growth responses were plantlet height (it was divided into four categories relatively based on the height of culture bottles (6 cm), which classified: 0 = dead, 1 = no growth, 2 = 1–2 cm, 3 = >2 to 4 cm, and 4 = >4–6 cm), number of shoots, number of leaves and number of roots. Anatomical observations were carried out on the 3rd to 5th leaves taken from the shoot apex. It was performed by calculating the number of stomata per microscope field of view and the length and width of the stomata. Chloroplast number was analyzed by counting the number of chloroplasts in the stomatal guard cells. Anatomical analysis was performed on 54 samples of mutants produced from selection under high temperature pressures. These samples were from 6 levels of gamma ray irradiation treatment (0, 10, 20, 30, 40, and 50 Gy) combined with 3 levels of temperature (25, 30, and 33°C). Each combination of treatments was repeated three times.

Data analysis of lethal dose was carried out using the CurveExpert 1.4 program and Microsoft Office Excel. Statistical data were conducted by using analysis of variance (Anova). Mean differences of single factors were analyzed with least significant difference (LSD) at 5% level using SPSS version 22.0 (IBM Corporation, Chicago, IL, USA) and Microsoft Excel. It was presented in a correlation (Pearson method) between temperature stress treatments of 33, 30, and 25°C (as a control) and observed variables, then analysed using Analyse Bivariate Correlations in SPSS version 22.0 (IBM Corporation, Chicago, IL, USA) and Microsoft Excel. The number of stomata was observed by counting the stomata visible in the field of view of a 4x10 light microscope. Stomata density was calculated using Microsoft Excel using the stomata density formula and stomata index. The calculation and measurement of stomata and the number of chloroplasts in guard cells were carried out using the ImageJ software. Stomatal density was calculated by using Microsoft Office Excel with the following formula:

$$\text{Stomatal Density mm}^2 = \frac{\Sigma \text{ Observed Stomata}}{0{,}19625} \text{x} 100\%$$

The mean and standard deviation of stomatal size, stomatal density, and number of chloroplasts in guard cells were calculated using SPSS version 22.0 (IBM Corporation, Chicago, IL, USA) and Microsoft Office Excel.

3.0 RESULTS AND DISCUSSION

Radiosensitivity of Bio Granola Potato Explants

The gamma ray irradiation doses affected the viability percentage of Bio Granola explants. The higher the dose, the more inhibited the rate of life (Table 1). The increased gamma ray irradiation doses also been reported to affect seed moisture content and wheat seed length [16], as well as the sensitivity of stevia plants [17]. This study showed that the combination of D48B-T treatment (terminal shoots of less than 1-year-old plantlets) and 20 Gy gamma-ray irradiation treatment caused higher viability rate (100%) than the control (95%) (Table 1).

There is a difference in response between terminal shoot and axillary shoots explants, where a lower survival percentage was obtained from axillary shoots, especially at doses of 30−50 Gy (Table 1). It was assumed to be related to the water content in plant cells or tissues. Axillary shoots are composed of older cells than the cells in the terminal shoots, so the vacuoles size in the axillary shoots is thought to be larger and contain more water. Factors affecting plant radiosensitivity can be divided into two main categories, environmental factors including atmosphere (oxic and anoxic), moisture content, post-irradiation treatment, and temperature. Meanwhile, biological factors include genetic differences, cell cycles, and nucleus and chromosomal volume [18]. High water content is one of the determining factors in the radiosensitivity response of plant tissues when exposed to irradiation [19]. The death process begins with yellowing of the tissues, followed by a change in color to brown or blackish, and then the tissue becomes dry. Similar symptoms also occur in *Stylosanthes hamata var. Verano* in the study of Ngoenngam *et al*. (2019) [20], which shows a change in the color of the apical meristem from green to yellow before entering the death stage due to the influence of gamma ray irradiation.

Table 1 Survival of Bio Granola potato plantlets of M_1V_1 population, at 4 WAC

Source of explants	Type of explant	Dosage (Gy)	Survival rate (%)	Equation	r	LD_{20}	LD_{50}
D48B	Terminal shoot	0	95	Polynomial fit: $Y=a+bx+cx^2+dx^3+ex^4$			
		10	87.5				
		20	100	$Y = 9.34 - 2.74x + 3.57x^2 - 1.41x^3 + 1.54x^4$	0.98	30.83	40.95
		30	72.5				
		40	52.5				
		50	50				
D48B	Axillary Shoot	0	90	Polynomial fit: $Y=a+bx+cx^2+dx^3$			
		10	87.5				
		20	90	$Y = 9.77 + 2.73x - 2.15x^2 + 2.65x^3$	0.97	22.61	32.53
		30	45				
		40	30				
		50	25				
D48L	Terminal shoot	0	97.5	Exponential fit: $Y=ae^{bx}$			
		10	97.5				
		20	87.5	$Y = 1.04 (-7.62x)$	0.83	33.88	95.58
		30	87.5				
		40	60				
		50	75				
D48L	Axillary Shoot	0	100	Sinusoidal fit: $Y=a+b*\cos(cx+d)$			
		10	92.5				
		20	65	$Y = 3.86 + 6.15 *\cos (4.38x-1.08)$	0.98	16.53	29.14
		30	60				
		40	12.5				
		50	0				

Note. The data was analyzed by CurveExpert 1.4, D48B: young motherstock (<1 year), D48L: old motherstock (>1 year)

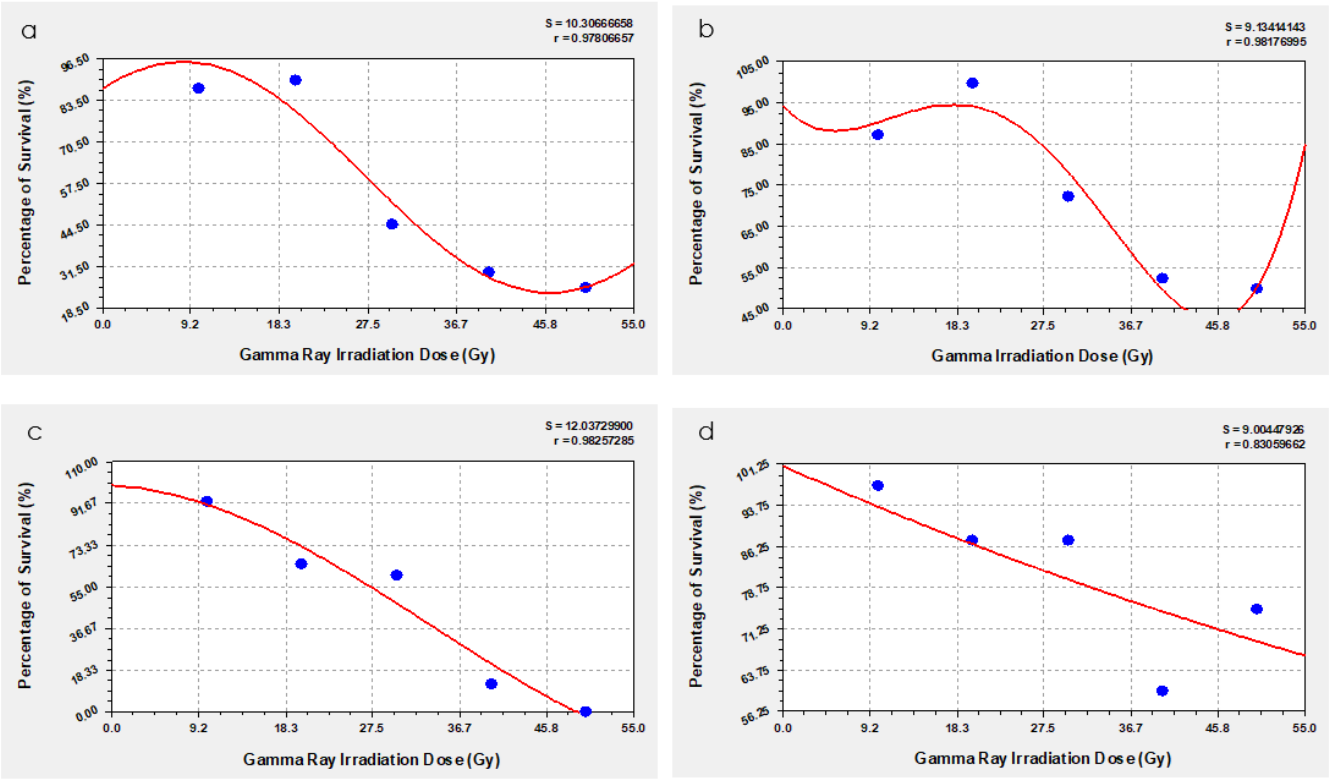


Figure 1 Radiosensitivity curve of Bio Granola potato explants to gamma irradiation based on CurveExpert 1.4 analysis, observed at 4 WAC: (a) young plantlet terminal shoots (D48B-T), and (b) young plantlet axillary shoots (D48B-A), (c) old plantlet terminal shoots (D48L-T), and (d) old plantlet axillary shoots (D48L-A)

The results of the curve fit equation analysis and the values of LD_{20} and LD_{50} are shown in Figure 1. The LD value is closely related to plant radiosensitivity, the plant sensitivity level resulting in morphological, physiological, and biochemical changes due to gamma ray irradiation. Based on LD_{20} and LD_{50}, axillary shoots were more sensitive than terminal shoots (Table 1). Differences in radiosensitivity values in different plants were also reported in the soybean variety of the Grobogan and Mutiara 1 [21], as well as in the seeds, plantlets, calluses, and *in vitro* shoots of *Indigofera zollingeriana* Miq [22] and shoots of *Stevia rebaudiana* Bert [17]. Gamma-ray irradiation has an important role in inducing mutations randomly. Although they are random, a range of doses can be used to increase genetic diversity.

Table 2 Morphological variations of M_1V_1 plantlets post-gamma-irradiation of Bio Granola *in vitro* shoots, at 4 WAC

Source of explants	Explant Section	Dosage (Gy)	Roots		Stems	Leaves	Shoots	
			R	RT	SC	LC	BSC	ABL
D48B	Terminal shoot	20	NHR	L	G	G	RG	W
		30	NHR	L	G	RG	Y	W
		40	NHR	S	B	RG	G	W
		50	NHR	S	Y	RG	RG	W
D48B	Axillary shoot	30	NHR	L	G	RG	G	B
		40	HR	S	Y	G	-	-
		50	-	-	Y	RG	-	-
D48L	Terminal shoot	20	NHR	L	G	RG	RG	W
		30	NHR	L	G	RG	Y	W
		40	NHR	L	G	RG	G	W
		50	-	-	G	G	RG	W
D48L	Axillary shoot	30	NHR	S	G	RG	G	B
		40	-	-	Y	RG	-	-
		50	-	-	Y	RG	-	-

Note D48B: young motherstock (<1 year), D48L: old motherstock (>1 year). Roots (R): non-hair roots (NHR) and hair roots (HR), root type (RT): long (L) and short (S), stem color (SC): green (G), yellow (Y) and brown (B), leaf color (LC): green (G) and reddish green (RG), bud stem color (BSC): green (G), reddish green (RG) and yellow (Y), arrangement of bud leaves (ABL): wide (W) and Bud (B)

Morphological Characteristics of Bio Granola Putative Mutants in M_1V_1 Populations

Gamma-ray irradiation exposure caused different responses, including changes in color, size, and stem type, leaves, and roots (Table 2). This is thought to be caused by a genetic mutation that affects the production of pigments in plant cells. In potatoes [23] and tomatoes [24], gamma ray irradiation also caused changes in leaf and flower color as well as stem type. Based on the results of this study, there was a difference response between terminal shoots and axillary shoots, where the higher variation was observed from the dose of 30−50 Gy treatments. Among the M_1V_1 populations, 30-50 Gy treatments caused chimerical effects or phenotype changes, which were reddish-green leaf color and reddish-green bud color. It was assumed that those chimeras were not yet separated, so they had to be sub-cultured to M_1V_4, which is expected to be solid.

Growth Responses of Bio Granola M_1V_1 and M_1V_4 Population

The interaction between factors on the growth of M_1V_1 and M_1V_4 population plantlets is shown in Table 3. The results of the multivariate fingerprint analysis showed that the irradiation dose factor had a significant effect on the growth response of the M_1V_1 and M_1V_4 populations. In the M_1V_4 population, interaction between three factors (gamma irradiation dose, explant source, and explant type) were significantly different to the shoot number variable. The height variable of the plantlet was influenced by the interaction between the explant source and the explant type, the irradiation dosage with the explant type, and the irradiation dosage with the explant source. The number of leaves was affected by the interaction between the radiation dose with the explant type, and the irradiation dose with the explant source. The root count variable was affected by the apparent interaction between the irradiation dose and the type of explant (Table 3).

Table 3 Recapitulation of three-factor interaction affecting the growth response of Bio Granola M_1V_1 and M_1V_4 plantlets post-gamma-ray irradiation treatments

Population	Character	D	S	E	D x S	D x E	S x E	D x S x E	Coefficient Variation (CV%)
M_1V_1	Height of the plantlet	**	tn	tn	*	tn	tn	tn	27.06
	Number of shoots	**	tn	tn	tn	tn	tn	tn	71.75
	Number of leaves	**	tn	tn	**	tn	tn	tn	28.81
	Number of roots	**	tn	tn	tn	tn	tn	tn	65.60
M_1V_4	Height of the plantlet	**	tn	tn	tn	**	**	tn	22.40
	Number of shoots	**	*	**	**	**	*	*	24.79
	Number of leaves	**	*	tn	*	**	tn	tn	26.13
	Number of roots	**	*	tn	tn	**	tn	tn	30.70

Note D = gamma-ray irradiation dose, S = explant source, E = explant type, * = significant on P<0.05, ** = strongly significant P<0.01, ns = not significantly different

The LSD test indicated that irradiation dose significantly influenced shoot number in the M_1V_1 population, whereas motherstock and explant type showed no significant effect. In contrast, all three factors of dose, motherstock, and explant type had significant effects on shoot number in the M_1V_4 population. For root number, similar patterns were observed in both M_1V_1 and M_1V_4 populations, except that explant type did not show a significant influence (Table 4).

Table 4 LSD test of three factors influencing the growth response of Bio Granola M_1V_1 and M_1V_4 plantlets post-gamma ray irradiation

Factor	Method application	Number of shoots		Number of roots	
		M_1V_1	M_1V_4	M_1V_1	M_1V_4
Dosage	0 Gy	$1.46^{a} \pm 3.40$	$1.29^{a} \pm 2.05$	$3.78^{a} \pm 1.20$	$3.10^{a} \pm 7.86$
	10 Gy	$1.15^{a} \pm 3.00$	$1.23^{ab} \pm 2.20$	$3.44^{a} \pm 1.68$	$2.89^{a} \pm 5.93$
	20 Gy	$1.12^{a} \pm 3.76$	$1.25^{a} \pm 3.65$	$2.63^{a} \pm 7.02$	$3.08^{a} \pm 0.45$
	30 Gy	$1.23^{a} \pm 4.00$	$1.17^{ab} \pm 2.40$	$1.55^{ab} \pm 1.10$	$2.85^{a} \pm 1.34$
	40 Gy	$0.53^{b} \pm 0.60$	$1.40^{a} \pm 1.60$	$0.30^{b} \pm 2.85$	$2.34^{ab} \pm 4.46$
	50 Gy	$0.28^{c} \pm 0.90$	$0.22^{b} \pm 0.35$	$0.05^{b} \pm 0.25$	$0.59^{b} \pm 1.50$
Source of explants	D48B	6.25 ± 6.40	$7.32^{a} \pm 3.72$	12.55 ± 17.61	$16.43^{a} \pm 10.29$
	B48L	5.27 ± 4.87	$5.81^{b} \pm 6.14$	10.97 ± 17.55	$13.29^{b} \pm 8.35$
type of explants	Terminal	6.16 ± 4.63	$7.69^{a} \pm 7.06$	12.16 ± 18.30	15.24 ± 12.59
	Axillary	5.36 ± 6.33	$5.43^{b} \pm 2.64$	11.36 ± 16.68	14.48 ± 6.28

Note The number followed by the same letter in the same column did not significantly different based on the 5% LSD test

Table 4 demonstrates increased variation in shoot number, whereas no such variation was observed in root number. This outcome may be attributed to the random nature of mutations induced by gamma-ray irradiation. Based on the LSD test in the M_1V_4 population, the highest number of shoots was obtained from 40 Gy treatment (1.40), the source of the explant D48B (7.32), the type of terminal explant (7.69), while the lowest number of shoots was obtained from 50 Gy treatment (0.22), the source of the explant D48L (5.81) and the type of axillary explant (5.43). The use of terminal shoots tended to increase the shoot number compared to the axillary shoots explant type. It was also reported by Royani *et al.* (2021) [22] on *in vitro* shoots of *Indigofera zollingeriana* Miq.

The differences appeared in potato plantlets after subcultured several times resulting in variations caused by the accumulation of genetic changes, cell selection and adaptation, epigenetics and gene regulation, changes in mutation stability, environmental adaptation and metabolism [25, 26].

The Effect of Heat Stress on the M_1V_4 Bio Granola Population

The correlation coefficient (r) ranged from 0.50 to 0.74, indicating a moderate to strong relationship among the characters, as shown by Pearson's analysis in Figure 2.

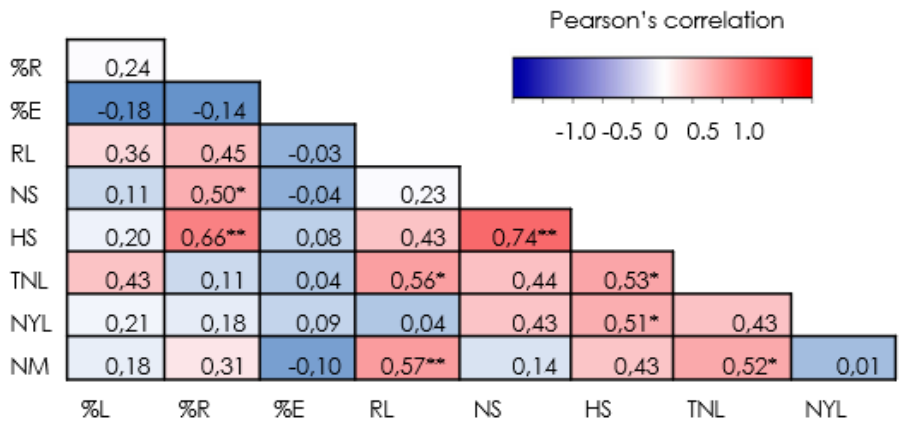


Figure 2 Correlation coefficient between heat stress temperature and putative mutant characteristics of Bio Granola; %L: percentage of life, %R: percentage of rooted, %E: percentage of etiolate, RL: root length, NS: number of shoots, HS: height of shoots, TNL: total number of leaves, NYL: number of yellow leaves, NM: number of microtubers

The results of correlation analysis using the Pearson method [27] showed a strong positive correlation between root percentage and total leaf count (0.709), root percentage and plantlet height (0.661), root length and total leaf count (0.556), root length and number of microtubers (0.605). The number of shoots and the number of microtubers (0.917) were correlated very highly. The number of yellow leaves and the number of microtubers (-0.757) were strongly negatively correlated. In line with the research of Long *et al*. (2024) [28], which suggests that more microtuber growth was associated with higher root activity, more consumption of the medium solute, and higher levels of sucrose transporter expression in the roots.

Anatomical Characteristics of the M_1V_4 Bio Granola Heat Tolerant

The findings showed that the combined treatment of gamma ray irradiation and high-temperature stress significantly affected microtuber formation in putative mutant plantlets (Table 5).

Table 5 The effect of heat stress on the growth of putative mutants, and the number of plantlets capable of forming microtubers as potential genotypes

Dose	Temperature	Growth (%)	Microtuber forming plantlet		Number of microtuber	
			Number	Percentage	Average	SD
0 Gy	25 °C	100	5	33.33	0.83	2.04
	30 °C	100	0	0	0	0
	33 °C	60	0	0	0	0
10 Gy	25 °C	100	3	20.00	0.33	0.82
	30 °C	100	1	6.67	0.50	1.22
	33 °C	40	0	0	0	0
20 Gy	25 °C	60	3	20.00	0.33	1.22
	30 °C	40	2	13.33	0.67	1.21
	33 °C	80	0	0	0	0
30 Gy	25 °C	100	0	0	0	0
	30 °C	100	2	13.33	0.67	0.94
	33 °C	80	0	0	0	0
40 GY	25 °C	100	0	0	0	0
	30 °C	100	3	20.00	1.00	1.41
	33 °C	80	0	0	0	0
50 Gy	25 °C	100	0	0	0	0
	30 °C	100	0	0	0	0
	33 °C	0	0	0	0	0

Note Total number of plantlets evaluated per treatment was 15 units

Table 5 indicates that temperature had a stronger effect than irradiation dose on both survival rate and microtuber formation. At 25 °C, all plantlets grew successfully (100%) across nearly all radiation doses, including the higher treatments of 40–50 Gy. At 30 °C, growth remained relatively high, ranging from 40–100%. However, at 33 °C a sharp decline in growth response was observed, particularly at doses ≥30 Gy, where plantlets failed to grow entirely (0%). These results highlight high-temperature stress as a major limiting factor for plantlet growth.

The results revealed that temperature had a strong influence on the percentage of plantlets forming microtubers. Rising temperatures inhibited microtuber development. In the control treatment (0 Gy at 25 °C), microtuber formation was observed in 33.33% of plantlets, but higher temperatures reduced this percentage. Radiation dose also affected microtuberization, at 10–20 Gy, microtubers formed at 25–30 °C with frequencies of 6.67–20%. At higher doses (30–40 Gy), microtuber formation declined. Plantlets exposed to 50 Gy failed to produce microtubers. At 33 °C, microtuber formation was completely suppressed across all treatments. These findings suggest that the combined stress of elevated temperatures and gamma radiation doses inhibit tuberization in potato.

Analysis of microtuber number showed that the highest value was obtained in the control treatment (0 Gy). However, variability was considerable, as reflected by the large standard deviation, which indicates heterogeneity within the plantlet tissues. The results indicate that heat stress combined with gamma irradiation acted synergistically to reduce plantlet growth and microtuber formation, with 33 °C identified as a critical limiting temperature. Favorable conditions for stimulating growth and microtuber development were observed at 25–30 °C under low to moderate radiation doses (0–20 Gy). These findings provide a useful basis for screening putative mutants under heat stress conditions. The combined treatment of gamma ray mutagenesis and *in vitro* selection has been successfully applied in rice, stevia, and tomato to enhance tolerance to abiotic stresses [6, 17, 24].

The results of the LSD test in Table 6 showed that gamma ray irradiation dose treatment had significant effects on the number of stomata, length of the stomata, the width of the stomata, and the number of chloroplasts. Those anatomical changes, particularly in chloroplast number, may contribute to variations in microtuber formation.

Tabel 6 Effect of Irradiation doses and temperature during *in vitro* selection on anatomical characters of Bio Granola putative mutants

Factor	Application method	Number of stomata			Length of stomata (µm)			Width of stomata (µm)			Number of chloroplasts		
		Average		SD	Average		SD	Average		SD	Average		SD
Dose	0 Gy	34.37^{a}	±	5.33	25.18^{b}	±	8.83	36.76^{a}	±	12.77	36.21^{a}	±	6.97
	10 Gy	34.93^{a}	±	10.11	36.13^{a}	±	12.14	41.52^{a}	±	19.36	37.02^{a}	±	5.80
	20 Gy	20.63^{b}	±	14.80	23.87^{b}	±	22.15	22.21^{b}	±	20.67	23.61^{b}	±	18.58
	30 Gy	29.74^{a}	±	7.06	24.94^{b}	±	8.90	41.79^{a}	±	21.52	34.29^{a}	±	7.11
	40 Gy	19.56^{b}	±	14.95	26.29^{b}	±	22.63	24.56^{b}	±	18.78	26.06^{b}	±	19.88
	50 Gy	20.37^{b}	±	16.40	21.02^{b}	±	18.13	20.60^{b}	±	17.11	18.34^{c}	±	14.14
Temperature	25 ^{O}C	32.58^{a}	±	9.25	41.93^{a}	±	8.46	22.81^{c}	±	8.32	30.99^{a}	±	7.04
	30 ^{O}C	28.32^{b}	±	14.12	21.84^{b}	±	14.71	37.47^{a}	±	20.41	30.80^{a}	±	15.27
	33 ^{O}C	18.91^{c}	±	13.97	14.94^{c}	±	11.68	33.44^{b}	±	23.00	25.98^{b}	±	19.02

Note The number followed by the same letter in the same column did not significantly different based on the 5% LSD test. SD: standard of deviation

Chlorophyll plays an important role in plant productivity, as chlorophyll is a key component in the process of photosynthesis. Research by Hutabarat *et al*. (2023) [29] suggests that high chlorophyll content often correlates with crop productivity. The parameter of the number of chloroplasts in guard cells is one of the most stable parameters in plant productivity. According to Kusumawati *et al*. (2020) [30], chloroplast calculation in stomata guard cells is an indirect and accurate method for evaluating the ploidy rate of plants. In general, the number of chloroplasts tended to increase as a plant grows. In addition, the average number of chloroplasts of 37.02 correlated positively with the size and openings of stomata of 36.13 µm (length) and 41.52 µm (width). Similar research by Posumah *et al*. (2017) [31], a larger number of chloroplasts allows for an increase in photosynthetic capacity and the availability of energy needed to support greater cell activity.

Microtuber formation obtained from control condition (without heat pressure) was significantly higher than in the heat stress conditions (Table 5). At 25 °C (control), the number of microtubers reached to 5 per bottle at a dose of 0 Gy (control). At 30 °C stress, putative mutants from the 40 Gy treatment yielded 3 microtubers/bottles. At 33 °C, all plantlets failed to form microtubers, and the putative mutants from the 50 Gy treatment had high mortality rates (80-100%).

4.0 CONCLUSION

Gamma ray irradiation combined with *in vitro* selection under heat stress successfully produced putative mutants of the potato cultivar Bio Granola with enhanced tolerance to elevated temperatures. The most effective treatment involved terminal shoot explants from young motherstock (less than one year old) exposed to irradiation doses of 20–30 Gy. This approach resulted in high survival rates, vigorous growth, and microtuber formation under 30 °C stress. In contrast, axillary explants and higher irradiation levels (≥40 Gy) led to poor survival, growth inhibition, and reduced microtuber production. Plantlets subjected to 50 Gy in combination with 33 °C stress experienced complete mortality.

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Conflicts of Interest

The authors declare that there is no conflict of interest regarding the publication of this paper.

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