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International Journal of Advanced Research in Food Science and Agriculture Technology

Journal homepage:
https://karyailham.com.my/index.php/fsat/index
ISSN: 3030-556X

Gamma-Irradiated M1V3 *Vanilla planifolia* Andrews Orchids: Growth Performance of *In-Vitro*

Basit Akolade Adigun[1,4], Homaa Faezah Moinuddin[1], Nabilah Ismail[2], Zaiton Ahmad[3], Rohayu Ma`Arup[1,*]

[1] Faculty of Food Science and Agriculture, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[2] Faculty of Science and Marine Environment, Universiti Malaysia Terengganu, 21030 Kuala Nerus, Terengganu, Malaysia
[3] Malaysian Nuclear Agency (Nuclear Malaysia), 43000, Bangi, Selangor, Malaysia
[4] Department of Science and Environmental Studies. The Education University of Hong Kong, Tai Po, New Territories, Hong Kong

ARTICLE INFO

Article history:
Received 11 July 2025
Received in revised form 15 August 2025
Accepted 20 August 2025
Available online 30 September 2025

Keywords:
Third generation; plant tissue culture; mutation breeding; gamma radiation; direct organogenesis

ABSTRACT

Vanilla planifolia is a commercially valued crop as it yields the natural vanilla flavour. However, due to its limited genetic diversity, hybrid production is difficult in *V. planifolia*. A feasible means to improve the *V. planifolia* genome is using gamma (γ) radiation. The M1V1 (10 Gy; First generation) *ex vitro* plant has improved increased vanillin concentration (μM) significantly ($P<0.05$) compared to control (0 Gy) in its leaves. Therefore, the objective of this study was to investigate the effect of γ radiation on the morphology of *in vitro*-generated M1V3 (mutation 1 in the third vegetative generation) *V. planifolia* plantlets. Initially, *V. planifolia* cuttings were exposed to four doses of gamma radiation (10, 30, 40, and 50 Gy) each with five replicates, and propagated *ex vitro* to generate M1V1 (first generation) and M1V2 (second generation) plants, followed by micropropagation of M1V2 explants. Morphological growth parameters like shoot height, root length, and number of leaves were recorded. There was only a significant difference ($p < 0.05$) in shoot height among the cultures at 10 days after culture (DAC), after which the height was not significantly different ($p > 0.05$) amongst the treatments throughout the experiment; 10 Gy was slightly higher. The increase in root per 10 DAC was significantly reduced in 10 Gy, while 30 Gy had the highest but insignificant compared to 0 Gy. Also, leaf number was significantly ($p < 0.05$) reduced in both 30 Gy and 10 Gy at 100 DAC. Summarily, lower-dose γ radiation has a mild effect on the M1V3 *V. planifolia* morphology of *in vitro* plantlets. To our knowledge, this is the first report on *in vitro* studies of M1V3 V. planifolia.

* *Corresponding author.*
E-mail address: rohayu_maarup@umt.edu.my

https://doi.org/10.37934/fsat.5.1.8899

1. Introduction

Vanilla planifolia Andrews, cultivated for its fruit, which yields the natural vanilla flavour, appeals to the international market for its applicability in foods, pharmaceuticals, and cosmetics industries [12,33]. This perennial monocotyledon plant which belongs to the family Orchidaceae, has been characterised with limited genetic variation [19,35]. Research on the evaluation of genetic diversity among populations of *V. planifolia* revealed no variation or extremely low variability [38]. Asexual reproduction methods are known to give rise to clones, which usually have very minute to null variation from their parents. The asexual means of propagation of the plant even from its native habitat, which served as the source of parent materials for most countries that adopted it for cultivation, greatly contributed to the invariability of its genome [8]. Likewise, traditional stem cutting propagation has been characterized as laborious and time-consuming [33,45] and pathologically and economically inefficient [38,45]. Cultivation through stem cuttings produces insufficient seeds, and seeds often have a high level of sterility [28,43]. An alternative means for rapid mass propagation of *V. planifolia* is *in vitro* culture [12], especially when dealing with clones of outstanding genetic profile and phytosanitary qualities [38].

Ionizing radiations such as X-rays and gamma rays can produce random deletions of chromosome parts and point mutations in plant cells [17]. Gamma rays can infiltrate the cells and affect the production of free radicals by atoms or biomolecules, and the generated radical species can destroy or facilitate the enhancement of important components [18]. Gamma radiation is employed in introducing mutations in plant propagules such as seeds, cuttings, pollen, or tissue-cultured calli [2,30]. Qualitative and quantitative traits of several crops belonging to cereals, legumes, oil seeds, vegetables, fruits, medicinal plants, ornamental plants, and fodder crops have been enhanced using mutation breeding [31]. A larger percentage of developed mutants are products of gamma radiation and, as a result, are prominently present in the list of registered mutants [20]. Crops having a restricted genetic base due to vegetative means of propagation are recommended for mutation breeding through mutagens (e.g., gamma radiation) to introduce genetic variation and improve desired traits [23]. Application of gamma radiation in *in vitro* cultures allows the development of multiple responses in plants, either to accelerate variation in species where it is difficult to obtain it with traditional breeding techniques or to promote the metabolic activities of the plant [34]. Combining gamma radiation treatment with *in vitro* culture technology can provide a better medium for the evaluation of mutants and ensure the preservation of traits of interest. *In vitro* mutagenesis in plant organs, tissues, and cell cultures provides the opportunity for the rapid and massive spread of mutant plants [37].

Ionizing radiation can produce a large number of lesions and needs to be totally integrated into the organism before it can be stable and become a part of it, Yunus *et al.,* [48] have attributed the slow regeneration rate of irradiated cultures of *Etlingera elatior* to intrasomatic competitions or diplontic selection among the cells in the meristematic tissue. Datta *et al.,* [6] highlighted the competition between the mutant cells and the ordinary cells, which determines the characters expressed in radiated plant tissues. The occurrence of chimerism in mutation breeding can be prevented by successive vegetative propagation of the mutants up to M1V4 and M1V5 generations [18]. Hermawati *et al.,* [16] expressed the genetic instability of M1V1 and M1V2 elephant grass, and screening of M1V3 for selection is obligated in a breeding programme.

Morphological variation as a result of gamma radiation was recorded in *Dendrobium odoardi* orchid [11]. Ellia *et al.,* [9] reported that *V. planifolia* cultures treated with 10 Gy radiation gave the best growth parameters when compared to the other doses, including the non-irradiated cultures (control). Hasim *et al.,* [15] reported that gamma treatment initiated physiological and morphological

variations in bananas and improved the growth parameters. Most *in vitro* studies of gamma-radiated *V. planifolia* are usually post-culture exposures to the mutagenic agent. In this research, we explored the *ex vitro* establishment of the mutants and maintained them for two generations (until M1V2) before generating the third generation (M1V3) through tissue culture techniques.

2. Materials and Methods

2.1 Plant Material and Gamma Radiation Treatment

Cuttings of Vanilla planifolia (containing 3 nodes) were irradiated using Biobeam GM 8000 (Gamma-Service Medical GmbH) at Agrotechnology and Biosciences Division, Malaysian Nuclear Agency, Bangi. The source of gamma radiation was caesium-137. The dosage used were 0 Gy, 10 Gy, 30 Gy, 40 Gy, and 50 Gy, and five cuttings per dosage were treated. These irradiated cuttings were cultivated under greenhouse conditions, maintained for 40 weeks per generation, and agronomical practices followed Ma'Arup *et al.,* [21]. Explants for *in vitro* culture were harvested from M1V2.

2.2 Sterilization

Sterilization followed Halim *et al.,* [14] with some modifications. Nodal segments with buds were rinsed under tap water prior to being washed in a conical flask containing tap water with a few drops of Tween-20 and then agitated frequently for 10 minutes. The explants were rinsed under running water for 7 minutes, followed by rinsing three times with sterile distilled water to remove the traces of tap water. Meanwhile, the laminar flow hood was surface sterilised by turning on the UV for 20 minutes and then thoroughly wiped with 70% (v/v) ethanol. Under the laminar flow cabinet, the explants were first sterilized by soaking in 70% (v/v) ethanol and shaking for 1 minute. Explants were transferred into a flask containing 0.1 M HgCl2 and constantly agitated for 5 minutes, then finally rinsed three times with sterile distilled water to remove the traces of the sterilant. Sterilized explants were then cultured individually on the prepared media.

2.3 Media Preparation

MS (Murashige and Skoog, 1962) media was supplemented with 30 g/L sucrose, 0.1 g/L myo-inositol, 1 mg/L benzyl aminopurine (BAP), 0.5 mg/L naphthalene acetic acid (NAA) [14]. The pH of the media was adjusted to 5.7 prior to the addition of 2.5 g/L of phytagel and the media was autoclaved for 20 minutes at 121^{o}C and 20 psi.

2.4 Growth Conditions

Cultured vials were incubated at 25 ± 2^{o}C under 16-hour photoperiods provided by 10-watt cool white, fluorescent lamps.

2.5 Subculturing

The same media component as above was used for subculturing, which was performed every 50 days after culture.

2.6 Statistical Analysis

For each treatment under study, shoot height and root length data were recorded every 10 days until 100 days after culture (DAC), and the number of leaves was recorded at 50 DAC and 100 DAC, and the accumulated data was used for statistical analysis. A two-factor analysis of variance compared the treatments by DAC for *in vitro* cultures. The significant values and means were separated at $p < 0.05$ by Duncan's Multiple Range Test (DMRT) using IBM SPSS version 20.0. The results presented are the mean and standard error.

3. Results

3.1. Morphology of In Vitro Generated M1V3 V. Planifolia

The treatments had insignificant ($p > 0.05$) effect on mean increase in shoot height per 10 DAC, and there was no significant interaction between the treatment and DAC (Table 1). The treatments significantly influence leaf number, but insignificant interaction was recorded between the treatments and DAC (Table 1). Root length of cultures per 10 DAC was significantly ($p < 0.05$) affected by the treatments and significant interaction was seen between the treatment and DAC (Table 1).

Table 1
Two-way analysis of variance of increase in shoot height, number of leaves and root length of V. planifolia cultures

Trait	Source	F	Sig.
Shoot Height	Treatment	2.62	0.08
	Days after culture (DAC)	1.98	0.05
	Treatment X DAC	0.75	0.76
Number of Leaves	Treatment	5.76	0.01
	DAC	9.64	0.00
	Treatment X DAC	2.18	0.13
Root Length	Treatment	9.73	0.00
	DAC	12.53	0.00
	Treatment X DAC	3.69	0.01

The average increase in height was significantly ($p < 0.05$) higher in 30 Gy (0.60) than 10 Gy (0.179) while the control (0 Gy) was insignificant with both 10 Gy and 30 Gy but was slightly higher than 0 Gy (Table 2 and Figure 1). Shoot height at both 50 and 100 DAC was insignificant ($p > 0.05$) amongst the treatments. However, 10 Gy was the highest and 30 Gy plantlets have the least. Leaf number was only significant ($p < 0.05$) amongst the treatments at 100 DAC, where the treatments significantly reduce the number of leaves (Table 2 and Figures 2 and 3).

Table 2
M1V3 V. planifolia shoot height (cm) at 10, 50, 100 DAC and number of leaves at 50 and 100 DAC

Treatment	Shoot Height (cm)			Number of Leaves	
	10 DAC	50 DAC	100 DAC	50 DAC	100 DAC
0 Gy	0.40 ± 0.75^{ab}	3.20 ± 0.78^{a}	5.37 ± 1.00^{a}	2.00 ± 0.30^{a}	4.80 ± 0.44^{a}
10 Gy	0.18 ± 0.09^{b}	3.50 ± 0.78^{a}	7.13 ± 2.01^{a}	1.20 ± 0.43^{a}	2.60 ± 0.62^{b}
30 Gy	0.60 ± 0.13^{a}	2.80 ± 0.95^{a}	4.50 ± 2.46^{a}	2.00 ± 0.68^{a}	2.50 ± 0.99^{b}

Different superscript alphabets in the same column shows significant difference ($p < 0.05$).

Average increase in height per 10 DAC for the first 100 DAC of M1V3 *V. planifolia* was insignificant ($p > 0.05$) amongst the treatments. Although it was slightly higher in 0 Gy (Table 3). The increase in

root length per 10 DAC in both the 0Gy (control) and 30Gy was significantly ($p < 0.05$) greater than the 10 Gy (Table 3). 30 Gy has the highest root growth but is insignificant when compared with the control.

Table 3
Increase in shoot height and root length per 10 DAC of micropropagated M1V3 V. planifolia for the first 100 days in culture

Treatment	Shoot Height (cm)	Root Length (cm)
0 Gy	0.62±0.05[a]	0.38±0.05[a]
10 Gy	0.45±0.07[a]	0.06±0.06[b]
30 Gy	0.42±0.11[a]	0.41±0.09[a]

Different superscript alphabets in the same column shows significant difference ($p < 0.05$).

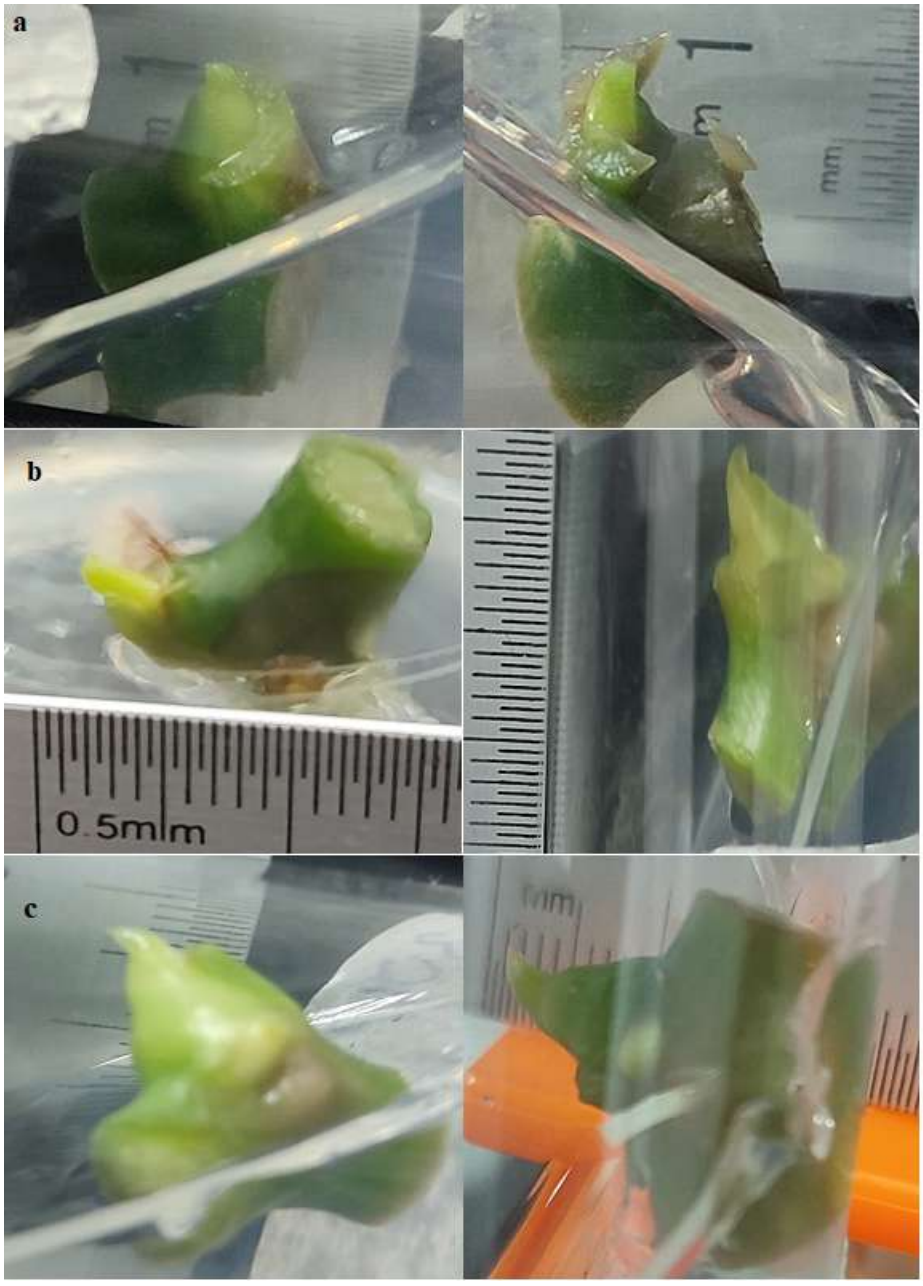

Fig. 1. M1V3 *V. planifolia* at 10 Days after Culture (a) 0 Gy (b) 10 Gy (c) 30 Gy.

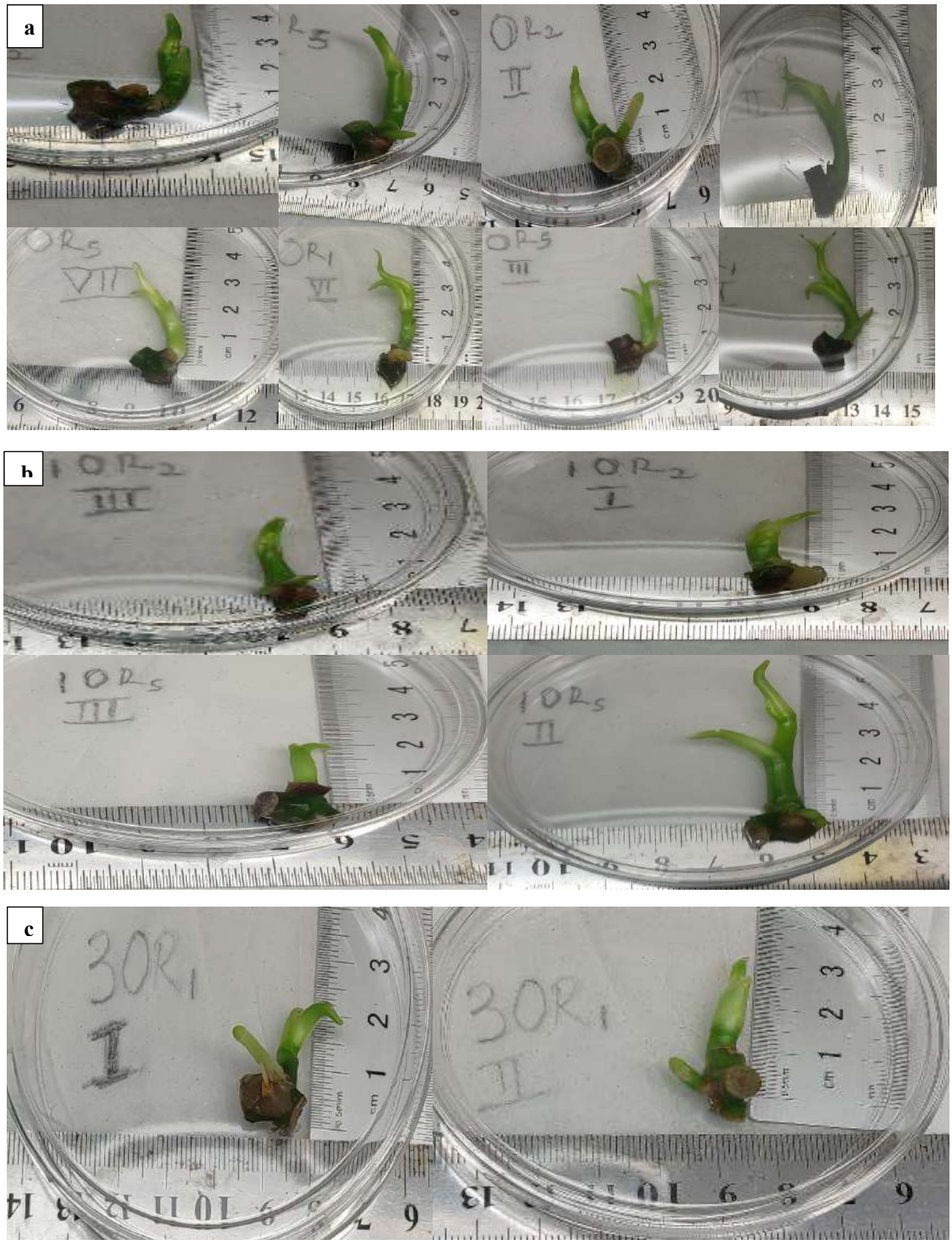

Fig. 2. M1V3 V. planifolia plantlets at 50 DAC (a) 0 Gy (b) 10 Gy (c) 30 Gy

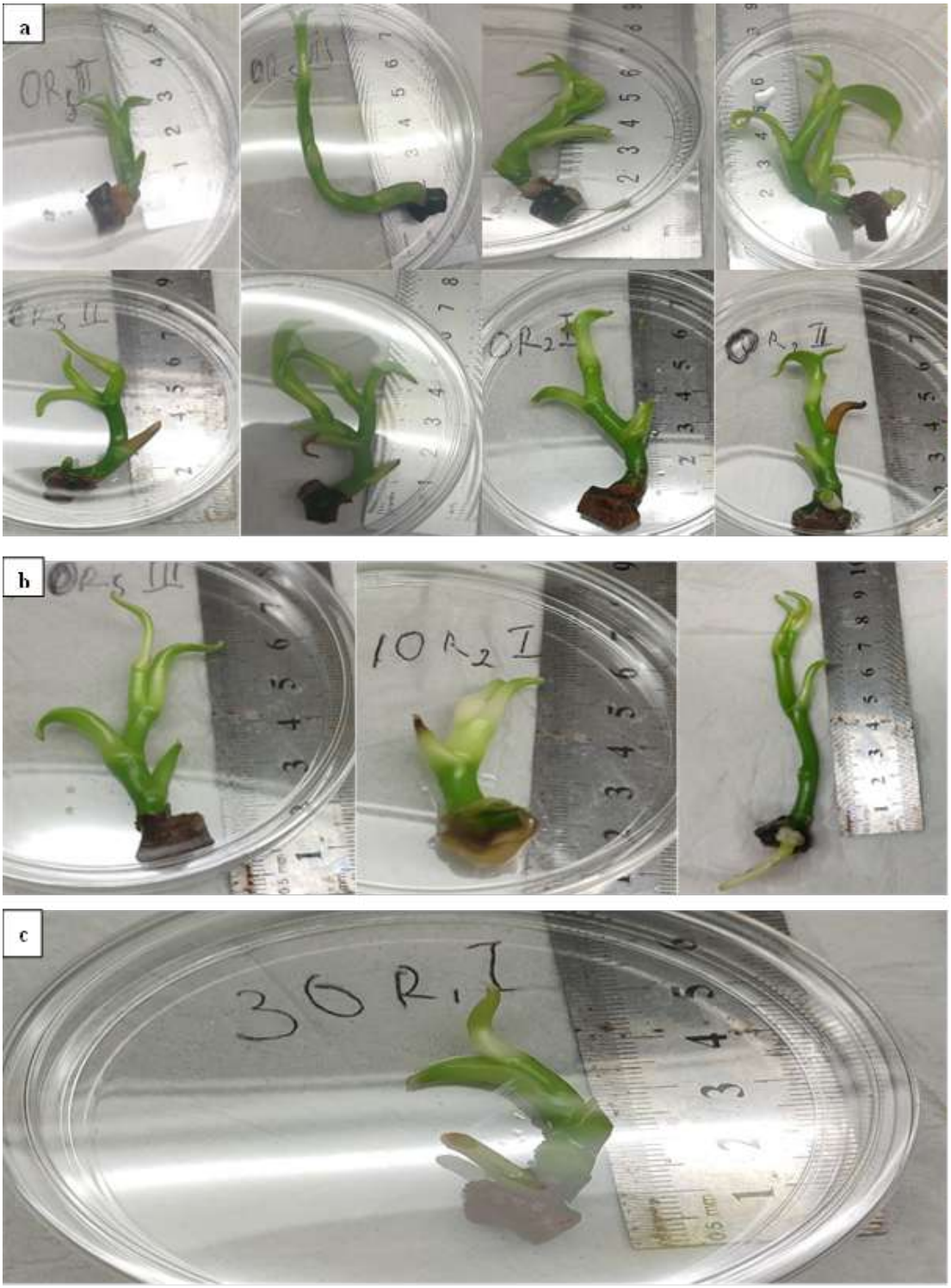

Fig. 3. M1V3 V. planifolia plantlets at 100 DAC (a) 0 Gy (b) 10 Gy (c) 30 Gy

4. Discussion

Lower dosage of gamma radiation doesn't have a basic fatal influence on the morphology of M1V3 *in vitro* generated *V. planifolia*. Irradiated culture shoot height was insignificant compared to the control; 30 Gy was slightly greater than the control, while 10 Gy had the least bud length at the early stage of growth (10 DAC). Higher shoot length in 10 Gy and lower in 30 Gy irradiated cultures, which were insignificantly different from the control in 50 DAC and 100 DAC were observed (Table

2). The insignificant influence of gamma radiation on shoot height may be because the plant has managed to incorporate the effect of the mutation in M1V1 and M1V2 and is only left with a mild and insignificant effect at M1V3. The major aim of mutation breeding programs is to produce a solid mutant plant that will have outstanding qualities or characters comparable to those of native cultivars. This is said to be achievable through subsequent successive propagation until later generations of the mutated tissue or cuttings, and this can be fast-tracked in a few months using tissue culture techniques [10,18,25]. The highest shoot height in 10 Gy M1V3 shows the relative stability of the irradiated cuttings and would support the fact that treated cuttings are being relieved of the negative effect of gamma radiation on the cuttings. The observation on shoot height could also be attributed to hormesis, where the lower dose (10 Gy) has stimulative effects, and the higher dose (30 Gy) proves inhibitive. Hormesis is said to occur when lower doses of a treatment have an encouraging effect and the higher doses have a retardation effect on living things [29]. The hormetic effect of gamma radiation on *V. planifolia in vitro* was reported, with doses exceeding 20 Gy (the minimum dosage applied) having growth-inducive influence and doses exceeding 20 Gy being inhibitory [35]. Likewise, Iglesias-Andreu *et al.,* [19]. reported hormesis and enhanced drought tolerance in gamma-radiated plantlets of *V. planifolia* Jacks. In addition, the findings of this research are similar to those of Billore *et al.,* [5], where an insignificant difference in shoot height between control and gamma irradiated M1V4 cultures of *Dendrobium sonia* under white light growth conditions was observed. However, significantly higher shoot height in 10 Gy and insignificant variation between the height of control and 30 Gy *V. planifolia* M1V1 cultures have been reported [9]. Mujar *et al.,* [26] reported significant shoot height retardation in 30 Gy irradiated cultures of *V. planifolia* M1V1 cultured plantlets.

However, post-irradiation cultures of banana (*Musa* spp.) resulted in an improved survival rate but retardation in shoot height [23]. Also, Shalini Udaya *et al.,*[36] reported stimulation of shoot growth from M1V1 irradiated at 5 Gy only, while all the subsequent vegetative generations until M1V4 had significantly lesser shoot height than the control in banana. In addition, Suraninpong and Wuthisuthimethavee [41] studied gamma irradiated *Anthurium andreanum* for four generations *in vitro* and reported that the control significantly exhibited the best morphology as compared to the irradiated plants.

A significantly low root length per 10 DAC was observed in 10 Gy plantlets, and there was no significant difference between 30 Gy and 0 Gy (Table 3). Root development of M1V3 *V. planifolia* wasn't uniform during the first 100 DAC in culture; some cultures didn't produce roots, and some roots stopped growing both in treated cultures and the control. The higher root length increase recorded in 30 Gy-treated plants demonstrated its stability, though insignificant when compared with the control, and this shows an enhancement of root growth by the gamma ray treatment. This outcome is similar to the research of Sri *et al.,* [40], where 30 Gy only encouraged root growth the most in *Vanda* (orchid) cultivated *ex vitro*. Also, a decrease in root growth *in vitro* due to radiation of 10 Gy or higher has been reported in some previous studies. Research on *Datura innoxia* revealed that 5 Gy enhanced root growth and produced greater root length as compared to the control, while higher doses declined root growth [3]. Common bean and mung bean treated with gamma radiation displayed significant reductions in shoot and root growth [44]. Generations M1V2 and M1V3 of *Etlingera elatior* shoot induction were affected by gamma radiation; development was morphologically similar to the control, and a reduced growth rate was observed [48]. Distinct root length retardation in 10 Gy treated V. planifolia contrasts with the report of Ellia *et al.,* [9], who reported significantly higher root length in the 10 Gy post-culture treatment of *V. planifolia* M1V1. Reduced root growth could be a result of the inhibitory effect of gamma radiation on the mitotic activities of the tissues or its perturbation of endogenous growth hormone levels [1].

Insignificant effects on leaf numbers were observed among both the irradiated and control plants at the early growth stage (50 DAC), but with time (100 DAC), the leaf number was significantly reduced by the radiation (Table 2). A similar trend in *V. planifolia* post-culture irradiation was reported, where a significantly lower number of leaves was generated [35]. Likewise, in black orchid *Coelogyne pandurate* culture, 5 Gy only stimulated leaf production, while higher doses, including 10 Gy, had an insignificant impact on leaf number [46]. Shoot height and leaf number of *Dendrobium Sonia* (orchids) were significantly reduced by gamma radiation doses, including 10 Gy [42]. Also, in *Phalaenopsis aphrodite* (butterfly orchids) culture treated with gamma radiation, leaf growth significantly decreased except for the minimal dose (10 Gy), which was insignificantly different from the control [22]. Basically, gamma radiation treatment has a negative influence on the growth and development of plants, and its effect may vary depending on the dosage and plant species being studied [47]. A dose-dependent decrease in the number of leaves and shoot height of *Zingiber officinale* in both M1V2 and M1V3 cultivated *ex vitro* has been reported [24]. Decreased shoot height and leaf weight with elevated chlorophyll contents were attributed to the post-culture gamma radiation of M1V4 of *Dendrobium sonia* [5]. Contrastingly, Iglesias-Andreu *et al.,* [19] discovered that a slightly higher dosage of gamma radiation promoted leaf number while the minimal dosage promoted shoot length in *V. planifolia* cultures. Using gamma rays and ethyl methanesulfonate (EMS) as mutagens on *Jasminum grandiflorum*, Ghosh *et al*., [13] reported an inverse relationship between the irradiated plant morphology and the dosage level of the mutagens. Gamma radiation on *ex vitro* M1V3 elephant grass stem cuttings increased leaf number and stems but had no effect on shoot height [16].

Characteristics observed in the third generation (M1V3) could be a more reliable result of the gamma radiation-mediated breeding of *V. planifolia*. No distinct damage or alteration was recorded, and this indicates that this generation of the plant is stable. It should be noted that during the first generation (M1V1) of this *V. planifolia* population, the control was morphologically greater than all the dosages administered in *ex vitro* conditions [21]. The outcome of this study implies that 10 Gy promoted shoot growth, although it was insignificant when compared to the control. However, the relative growth inhibition seen in root length and leaf number may be due to the interaction of the radiation with growth hormone synthesis in the plant. Dehgahi and Joniyas [7] have reported that the consequences of gamma radiation exposure on plants may include a reduction of growth-promoting factors such as cytokinin, an increase in fresh weight, and a delay in germination periods. In living tissues, gamma irradiation could affect proteins by causing spatial changes in their molecular structures, amino acid oxidation states, distortion of covalent bonds, and free radical formation [32]. In addition, M1V1 (10 Gy) *ex vitro* plant have improved increased vanillin concentration (μM) significantly ($P<0.05$) compared to control (0 Gy) in its leaves [4].

5. Conclusion

This research explored the use of plant tissue culture techniques to regenerate M1V3 *V. planifolia*. The outcome doesn't reflect any morphological damage to the generated plants. The absence of retardation in growth parameters depicts the stability of the irradiated plants, and it is expected that in the next one or two generations (M1V4 or M1V5), the treated plants may have an outstanding growth performance.

Acknowledgement
This research was financially supported by Universiti Malaysia Terengganu through the Talent and Publication Enhancement Research Grant (UMT/TAPE-RG/2020/55278). We would like to appreciate Amani Vanilla Temerloh for providing the plant materials for this study and Mr. Shuhaimi Shamsudin from the Malaysian Nuclear Agency for irradiating the samples.

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