

Electron Beam Induced Mutation in *Curcuma longa* L. Against Bacterial Wilt Disease

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Received: 30 January 2024, Revised: 14 March 2024, Accepted: 21 March 2024, Published: 10 September 2024

Abstract

Curcuma longa L. is a medicinal plant that contains bioactive constituents with various pharmacological properties. However, turmeric is vulnerable to the bacterial wilt disease caused by *Ralstonia solanacearum*, substantially lowering yields and resulting in death. Thus, this study aims to induce mutations of turmeric cv. 'Trang 2' by applying electron beams (8 MeV) and selecting the resulting populations resistant to bacterial wilt disease. Sixty-day-old plantlets cultured *in vitro* were exposed to 0, 50, 100 and 150 Gy electron beams with a 240 Gy/min dose rate. The experiment was performed at the Thailand Institute of Nuclear Technology. To explore the potential of electron beam sensitivity in the survival and growth rate, unirradiated plantlets were used to normalize the radiation treatments. Plantlet survival was used to calculate the lethal dose (LD), and the number of new shoots was used to estimate the growth reduction (GR) dose through regression analysis.

The survival and growth rates of the plantlets decreased as the radiation dose increased. At doses of 100 and 150 Gy, the turmeric plantlets could not produce new shoots. The median lethal dose (LD₅₀) was 58.6 Gy. A 50 % growth reduction dose (GR₅₀) was observed at 45.3 Gy. Symptom severity ranged from 11.7 to 91.7 %, demonstrating significantly lower levels in the EB RTP-2 and EB RTP-4 M₁V₅ electron-beam-irradiated populations. Furthermore, the disease incidence was 33.3 % in the EB RTP-6 population. Finally, turmeric populations resistant to bacterial wilt (EB RTP-2, EB RTP-4 and EB RTP-6) were isolated after electron-beam-induced mutation at 50 Gy.

Keywords: Bacterial wilt disease, Electron beam, Induce mutation, Tissue culture, Turmeric

Introduction

Curcuma longa L. is a member of Zingiberaceae, also called as “Turmeric”. It is a popular medicinal herb in Thailand and also used as a spice and a coloring agent. According to Thai traditional medicine, fresh and dried rhizomes can be used for peptic ulcer treatment, as carminatives, for wound treatment and as an anti-inflammatory agent [1]. Previous studies found the turmeric rhizome to be a valuable source of antioxidant compounds, and it has been incorporated in many health products [2]. Moreover, turmeric is an important ingredient for Thai food, using both mature and young rhizome stages. Turmeric cv. Trang 2 has been reported to contain an average 11.04 % of curcuminoids and 7.78 % of essential oil, which are higher than the Thai herbal medicinal standard [3]. One of the problems of turmeric production is bacterial wilt disease caused by *Ralstonia solanacearum* which is capable of infecting young plants, thrives mostly in humid and hot climates. It leads to a substantial reduction in yield of turmeric and impacts both economic and agricultural aspects [4]. Disease symptoms first appear as sudden wilted, curled, yellowed leaves, eventually leading to the death of the whole plant [4]. *R. solanacearum* is a soil-borne bacterium that causes bacterial wilt disease in a wide range of host plants that affects over 450 plant species [5]. This disease is of serious concern in vegetable cultivation worldwide, leading to severe yield losses. Various research attempts have been made to control this disease, but with limited success due to the complex nature of the pathogen [6].

The chromosome number of turmeric is $2n = 3x = 63$ [7]. It is propagated by rhizomes owing to a high density of sterile pollen. Mutation breeding is a technique using ionized radiation (gamma ray, x ray, ion beam, electron beam) and chemical mutagens to induce genetic variation in plants to improve or develop new crop varieties with good traits, such as high yield, disease resistance and tolerance to abiotic stresses, according to the desired purpose [8]. It was reported that mutagenesis has been used to create plant varieties, such as rice, barley, maize, wheat, beans and green peas, that show resistance to pathogens [9]. Mutant variety of *Citrus reticulata* was developed from budwoods of local Kinnow irradiated at 20 Gy of gamma rays that showed moderate to high resistance to citrus canker, scab and wither-tip diseases [10]. Electron beam-induced mutagenesis is a method used to generate genetic diversity in plants, and this method has many outstanding advantages: Lower damage rate; higher mutation rate and a wider mutational spectrum [11]. In addition, plant tissue culture is advantageous for inducing mutation as it allows for easy handling of numerous plants and facilitates the separation of mutated cells from non-mutated ones [12]. To date, there are not many studies on the effects of electron beams in turmeric for inducing mutations. Therefore, this research aims to explore the potential of 8 MeV electron beam-sensitivity on survival and growth rate of turmeric *in vitro* culture, providing a baseline of optimal dose utility for turmeric mutation breeding and selection of turmeric against bacterial wilt disease.

Materials and methods

Bacterial strain and growth conditions

A stock culture of an *R. solanacearum* isolate was obtained from the Plant Protection Research and Development Office at the Department of Agriculture of Thailand and used in the experiment. The bacterial isolate was streaked on nutrient agar (NA) and incubated at 30 °C for 48 h. A single colony of the isolate was then selected and cultured on triphenyl tetrazolium chloride (TZC) at 30 °C for 48 h. *R. solanacearum* cells were suspended in nutrient broth (NB) and adjusted to approximately 10^8 CFU/mL, determined using a spectrophotometer at 600 nm. The suspension was used to inoculate turmeric samples.

Plant materials and inoculation methods

Intact buds of field-grown turmeric cv. ‘Trang 2’ were surface-disinfected in sodium dichloroisocyanurate (1,000 mg/L) for 30 min and rinsed 3 times with sterile deionized water. Explants were obtained and cultured on an MS medium [13] for 60 days. Subsequently, the 60-day-old plantlets were grown on an MS medium supplemented with 2 mg/L benzyladenine (BA) as a growth regulator. The samples were sub-cultured on a hormone-free MS medium containing sterilized 2.5 mg/L Kelcogel gellan gum and 30 g/L sugar. The pH of the medium was adjusted to 5.8 before placing the samples in the autoclave. The cultures, kept under conditions of 16 h of light, 25 ± 1 °C, and 65 - 70 % relative humidity, were prepared for exposure to electron beams.

Pathogenicity was tested using 60-day plantlets cultured on a hormone-free MS medium. The well-rooted plantlets were transferred *ex vitro* for acclimatization and hardening. The plantlets were then washed with water to remove excess agar attached to their roots, air-dried for 10 min, and planted in small pots (10×7 cm) containing peat moss. The potted plants were kept in a greenhouse under controlled conditions for 2 months. Subsequently, the plants were inoculated by immersion of their wounded roots in the bacterial (*R. solanacearum*) cell suspension for 24 h [14] and transferred to pots containing soil and leaf mold (1:1 ratio).

Electron beam *in vitro* mutagenesis and selection of turmeric resistant to bacterial wilt disease

Sixty-day plantlets cultured *in vitro* were exposed to 0, 50, 100 and 150 Gy electron beams, using a high-energy electron accelerator. This experiment was performed at the Thailand Institute of Nuclear Technology. The beam had an energy of 8 MeV, a current of 100 mA, a dose rate of 240 Gy/min, a conveyor speed of 1.55 m/min, and pulse repetition frequencies (PRF) of 10 Hz (50 Gy), 20 Hz (100 Gy) and 30 Hz (150 Gy). To calculate the survival and growth rate, unirradiated plantlets were used to normalize the results of the radiation treatments. Plantlet survival was used to calculate the LD, and the number of new shoots was used to estimate the growth reduction (GR) dose through regression analysis [11,15].

To assess the tolerance of the turmeric mutants against *R. solanacearum*, the plantlets were sub-cultured on new media until the M₁V₂ and M₁V₃ generations. This process was conducted at a regular interval of 2 months. M₁V₄ was achieved by clonal propagation from M₁V₃ individual plantlets, maintaining individual clones until the M₁V₅ generation. After 10 months of *in vitro* growth post-irradiation, the irradiated and non-irradiated (control) plantlets were transferred to small pots containing peat moss for *ex vitro* acclimatization and hardening. Two months after transplanting, 8 M₁V₅ populations with 3 replications and 6 plants per replication were inoculated by immersing their wounded roots in the bacterial cell suspension for 24 h. The same inoculation method was used for the non-irradiated control.

Symptom severity was assessed by determining the proportion of wilted leaves 14 days after inoculation, using the following scale [16,17]: 0 = no wilt symptoms, 1 = 1 - 25 % of leaves wilted, 2 = 26 - 50 % of leaves wilted, 3 = 51 - 75 % of leaves wilted and 4 = 76 - 100 % of leaves wilted.

The disease incidence (%) was calculated using the formula below [16,17]:

$$\text{Disease incidence (\%)} = \frac{\text{The number of plants with wilt symptoms}}{\text{The total number of plant samples}} \times 100. \quad [1]$$

Statistical analysis

The data were subjected to an analysis of variance (ANOVA) with a completely randomized design (CRD) and 3 replications per treatment. Significance was set at $p < 0.01$. A least significant difference test

(LSD) was performed to separate the means when significant effects were detected. The standard error (SE) was calculated to compare the results from the 3 replicate experiments.

Results and discussion

Electron beam sensitivity of turmeric

The results of the electron beam-sensitivity experiment were evaluated regarding the number of surviving plants and the number of new shoots, expressed as survival and growth rates, respectively, 30, 45 and 60 days after exposure (DAE). The determination of radio sensitivity is a prerequisite for large-scale irradiation for inducing mutations in plants [18]. Each part of plants including each plant genotypes have sensitivity from irradiation; a test of radio sensitivity is fundamentally used as a noticeable effect of radiation [11,19]. Two groundnut genotypes were generated with 10 MeV linear accelerator facility for low dose application (0.1 - 1 kGy) in pulse mode using unscanned scattered beam for irradiation of groundnut seeds, 50 % growth reduction (GR_{50}) dose was standardized in 5 groundnut genotypes and were significant differences for radio-sensitivity among these genotypes [11]. The effect of a mutagen on plants is verified by their survival rate and the level of regeneration and multiplication [18]. The survival rate is one of the key attributes examined in any *in vitro* mutagenesis experiment because plant mortality indicates the extent of damage caused by exposure of the seedlings to any ionizing radiation including electron beams [15]. The survival rates of turmeric plantlets at 30, 45 and 60 DAE as a percentage of the non-irradiated control are shown in **Table 1**. Doses of 50, 100 and 150 Gy resulted in significant differences ($p < 0.01$) at 30, 45 and 60 DAE compared to the control, except for the plants subjected to 50 Gy, 30 DAE. The survival rate decreased as the DAE and the radiation dose increased (**Table 1**). Similar results have been reported for electron-beam-irradiated *in vitro* cultures of *Curcuma alismatifolia* [17]. Electron beams exhibit a low linear energy transfer (LET) of approximately 0.2 KeV/ μ m [20], which is administered as short pulses of radiation, resulting in only slight physiological damage to the M_1 generation but a high mutation frequency in the M_2 generation [21].

Table 1 Survival and growth rates of turmeric plantlets cultured *in vitro* and exposed to different electron beam irradiation doses.

Treatment	Survival rates			Growth rates		
	30 DAE	45 DAE	60 DAE	30 DAE	45 DAE	60 DAE
Control	100.00 ^{a1/}	100.00 ^a	100.00 ^a	100.0 ^{a1/}	100.0 ^a	100.0 ^a
50 Gy	97.62 ± 8.3 ^a	54.76 ± 4.1 ^b	40.48 ± 8.3 ^b	17.26 ± 2.6 ^b	21.86 ± 5.9 ^b	23.26 ± 3 ^b
100 Gy	65.76 ± 3.7 ^b	34.24 ± 3.7 ^c	12.43 ± 5 ^c	0.00 ^c	0.00 ^c	0.00 ^c
150 Gy	75.56 ± 7.7 ^c	24.44 ± 7.7 ^d	6.67 ± 11.6 ^c	0.00 ^c	0.00 ^c	0.00 ^c
F-test	**	**	**	**	**	**
C.V. (%)	5.59	8.88	16.6	4.4	9.7	4.9

Note: The data represent means ± standard error. Different lowercase letters in the same column indicate significant differences at $p > 0.01$ by the LSD test.

The radiosensitivity test revealed a substantial decline in the number of new shoots with the increase in the radiation dose. At doses of 100 and 150 Gy, the turmeric plantlets could not produce new shoots. Compared to the control, the 50 Gy dose resulted in significant 5.8-, 4.6- and 4.3-fold reductions in growth ($p < 0.01$) at 30, 45 and 60 DAE, respectively (**Table 1**). Electron beams cause gene mutations by damaging

the single- (SSB) and double-strand breaks (DSB). Nevertheless, they have little influence on the function of the cell wall, plasma membrane, or cellular protein [11]. The physical processes by which these low-energy electrons interact with matter are unclear. High relative biological effectiveness (RBE) was observed at an energy level of 8 MeV with a high-dose-rate electron beam (240 Gy/min). A study reported that gamma rays at doses of 40 and 60 Gy reduced the growth rate of 60-day turmeric plantlets to less than 50 % that of the non-irradiated control [22]. Several studies have reported the effects of radiation on cell division and the inhibition of plant growth provoked by the destruction of the meristem [23,15]. In addition, electron beams generate root abnormalities because of DNA damage to the root tissue, resulting in a high percentage of abnormal seedlings and a decrease in their growth rates as demonstrated by irradiation of *Lathyrus chrysanthus* Boiss; however, gamma irradiation of the plantlets under *in vitro* conditions did not yield such effects [24].

Determination of lethal (LD) and growth reduction (GR) doses

The 25 % (LD₂₅), 50 % (LD₅₀) and 75 % (LD₇₅) LDs were estimated for turmeric samples irradiated with electron beams *in vitro*, using the regression equation of the survival rate as a percentage of the control (**Figure 1**). The LDs were 99.2 Gy (LD₂₅), 58.6 Gy (LD₅₀) and 18.0 Gy (LD₇₅). The median growth reduction (GR₅₀) was calculated using the regression equation of the growth rate as a percentage of the control (**Figure 2**). Identical regression equations were used to evaluate the GR₂₅ and GR₇₅. Doses of 84 Gy (GR₂₅), 45.3 Gy (GR₅₀) and 6.7 Gy (GR₇₅) resulted in growth reduction in the turmeric irradiated *in vitro*. This information will provide a baseline for optimizing the radiation dose in a turmeric mutant breeding program. A previous study reported that the optimal electron beam dose for plantlets of the *Curcuma* hybrid 'Laddawan' cultured *in vitro* should be less than 50 Gy [15].

The optimal dose for plant mutagenesis is identified according to the number of detectable effects on the irradiated samples [25]. The optimal dose frequency should induce a mutation with minor unintended damage. Moreover, various physical, chemical and biological factors can alter the radiation effects in plants. The maximum probability of generating novel mutants for crop improvement is accomplished at a 50 % lethal dose (LD₅₀) or when 50 % of the plants experience growth reduction (GR₅₀) [26]. The LD₅₀ and GR₅₀ are considered adequate because lower doses produce slight effects on the plant genome that may result in only morphological changes, whereas, higher doses may result in numerous changes to the genome, leading to undesirable mutations [26]. However, the LD₂₅ and LD₇₅ have also been reported to be effective [27,28].

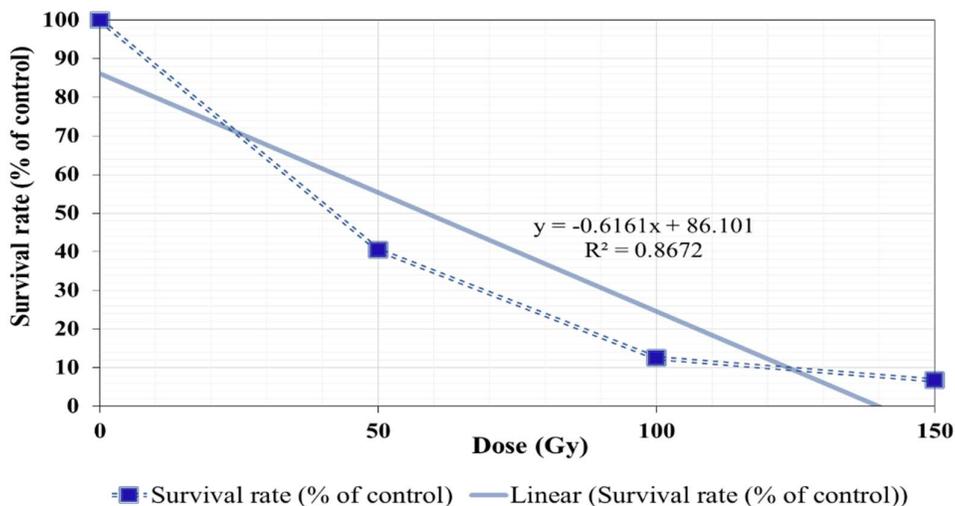


Figure 1 LD determination and regeneration equation for turmeric irradiated *in vitro* with different electron beam doses 60 DAE.

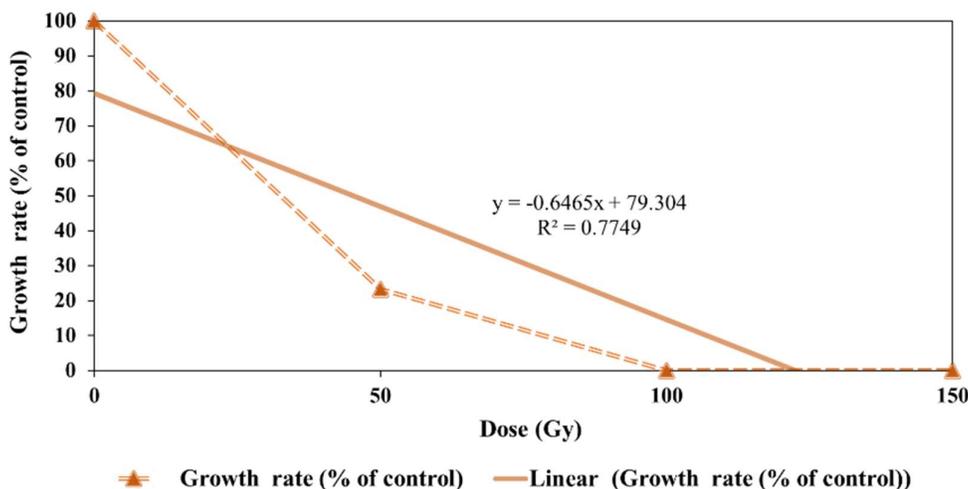


Figure 2 Growth reduction and regeneration equation of turmeric irradiated *in vitro* with different electron beam doses 60 DAE.

Selection of turmeric mutants resistant to bacterial wilt disease

The well-rooted irradiated and non-irradiated plantlets of the M₁V₅ generation were acclimatized and hardened *ex vitro* in peat moss. Sixty days after transplantation, plants were inoculated by immersion of their wounded roots in the bacterial cell suspension for 24 h, and transferred to pots containing soil and leaf mold (1:1 ratio) [14]. Plants were evaluated daily for wilt symptoms and disease incidence. When the disease developed, brown and discoloration was observed on the leaves. Wilt symptoms initiated at the apex of the plant and moved towards the base. Wild type turmeric exhibited wilting symptoms within 2 - 14 days after inoculation, finally resulting in plant deaths. Hence, turmeric mutants resistant to bacterial wilt disease were selected at 14 days after inoculation. The workflow of electron-beam-induced mutagenesis in turmeric cv. ‘Trang 2’ is depicted in **Figure 3**.

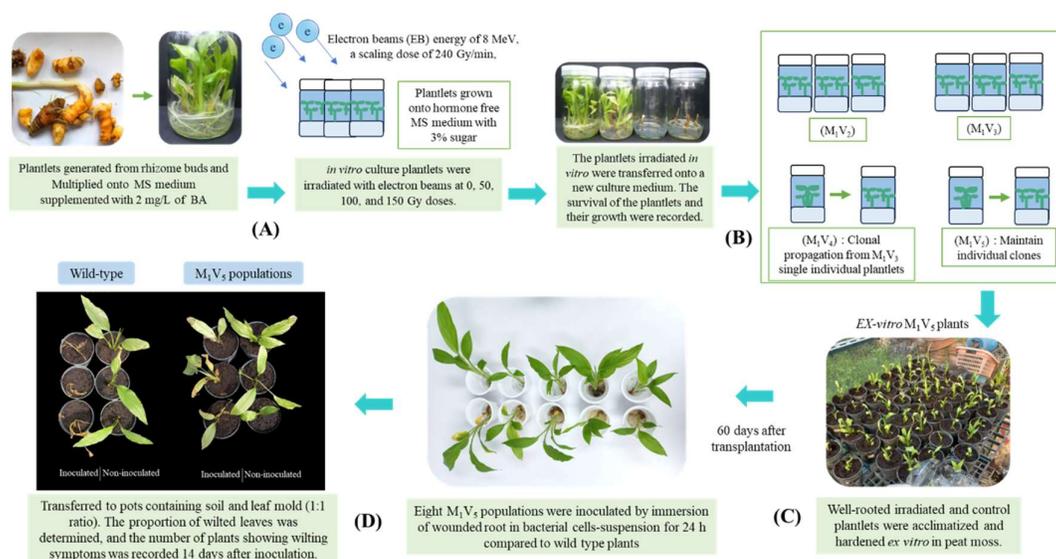


Figure 3 Workflow of electron-beam-induced mutagenesis in turmeric cv. ‘Trang 2’. (A) Plantlets generated from rhizome buds and cultured *in vitro* were irradiated with electron beams at 0, 50, 100 and 150 Gy doses. (B) The plantlets irradiated *in vitro* were transferred onto a new culture medium and grown in a chamber at 25 ± 2 °C and 65 - 70 % relative humidity with a 16-h photoperiod. The survival of the plantlets and their growth were recorded. The plantlets were sub-cultured in a fresh medium until the M_1V_5 generation. (C) Well-rooted irradiated and control plantlets were acclimatized and hardened *ex vitro* in peat moss. (D) Sixty days after transplantation, plants were inoculated by immersion of their wounded roots in a bacterial (*R. solanacearum*) cell suspension for 24 h and transferred to pots containing soil and leaf mold. The proportion of wilted leaves was determined, and the number of plants showing wilting symptoms was recorded 14 days after inoculation.

The results revealed that symptom severity (%) and disease incidence (%) varied after the roots were dipped in the *R. solanacearum* suspension for 24 h and the plants were grown in soil for 14 days. The symptom severity ranged from 11.7 to 91.7 % and was significantly lower in the EBRTTP-2 and EBRTTP-4 M_1V_5 electron-beam-irradiated populations, whereas the disease incidence was 33.3 % in the EBRTTP-6 population. Among the 8 irradiated populations, EBRTTP-6 was shown to be resistant as it displayed symptom severity and disease incidence lower than 50 %. EBRTTP-2 and EBRTTP-4 were moderately resistant because they showed minimal symptom severity; however, the incidence of the disease was 60 and 50 %, respectively. On the other hand, EBRTTP-3 and EBRTTP-5 were susceptible owing to their high disease incidence. The remaining irradiated populations were highly susceptible (**Table 2**). The symptoms initiated as leaf curling followed by wilting of the plants within a few days, which led to their deaths. The pathogen infects the roots of susceptible plants, usually through wounds [29]. Colonization of the xylem by the bacterium prevents water from moving to the upper portion of the plant [16]. Here, we performed artificial inoculations by immersing the wounded roots in bacterial cell suspensions. We have previously performed inoculations by injecting a bacterial cell suspension at the base of the stem and infecting soil with an *R. solanacearum* cell suspension (data not shown). The symptoms of wilting caused by *R. solanacearum* infections are influenced by the pathogenic strains, inoculation methods, and environmental factors such as temperature and humidity [16,29].

Table 2 Symptom severity and disease incidence percentages after the roots of turmeric plants were dipped in an *R. solanacearum* suspension for 24 h and the plants were grown on soil for 14 days. Wild-type turmeric and 8 electron-beam-irradiated M₁V₅ populations were tested.

Populations	Symptom severity (%)	Disease incidence (%)
Wild type	91.7 ± 30.6 ^a	83.3 ± 15.3 ^a
EBRTP-1	58.3 ± 7.6 ^{bc}	75.0 ± 29.7 ^{ab}
EBRTP-2	11.7 ± 5.8 ^d	60.0 ± 6.9 ^{bc}
EBRTP-3	35.0 ± 8.7 ^{cd}	75.0 ± 31.9 ^{ab}
EBRTP-4	11.7 ± 2.9 ^d	50.0 ± 11.6 ^c
EBRTP-5	40.0 ± 30 ^{cd}	80.0 ± 22.3 ^a
EBRTP-6	15.0 ± 13.2 ^d	33.3 ± 6.1 ^d
EBRTP-7	85.0 ± 5 ^{ab}	80.0 ± 9.1 ^a
EBRTP-8	55.0 ± 35 ^{bc}	75.0 ± 15.8 ^{ab}
F-test	**	**
C.V. (%)	40.3	25.2

Note: The data represent means ± standard error. Different lowercase letters in the same column indicate significant differences at $p > 0.01$ by the LSD test.

Although the mechanisms of action of electron beams are still unclear, their mutagenic effects are well-known. For instance, electron beam irradiation induced mutation in *Arabidopsis thaliana* by causing DNA damage similar to C⁶⁺ ions [30]. Thus, electron beams show the potential for generating mutations and have been used in several plant species, including rice, ornamentals, cereals, legumes, fiber crops, cluster beans and groundnuts [15,31-34]. Based on cowpea chlorophyll mutants, a chemical mutagen (EMS) seemed to be the most effective (6.47 %) and efficient mutagen (27.09 %), followed by electron beams and gamma rays [34]. Electron beams can be used as mutagens for crop improvement because they are highly effective [11], cheap and safe [34]. One of the main objectives of future crops production is to broaden-spectrum resistance to pathogenic disease. Mutagenesis enables the identification of novel genes associated with disease resistance beneficials especially when there is no reliable source of resistance found in the nature that makes it impossible to introduce to susceptible cultivars by hybridization [10]. In this study, turmeric populations showing moderate and high resistance (EBRTP-2, EBRTP-4 and EBRTP-6) to bacterial wilting disease were obtained by electron-beam-induced mutations (**Figure 4**).

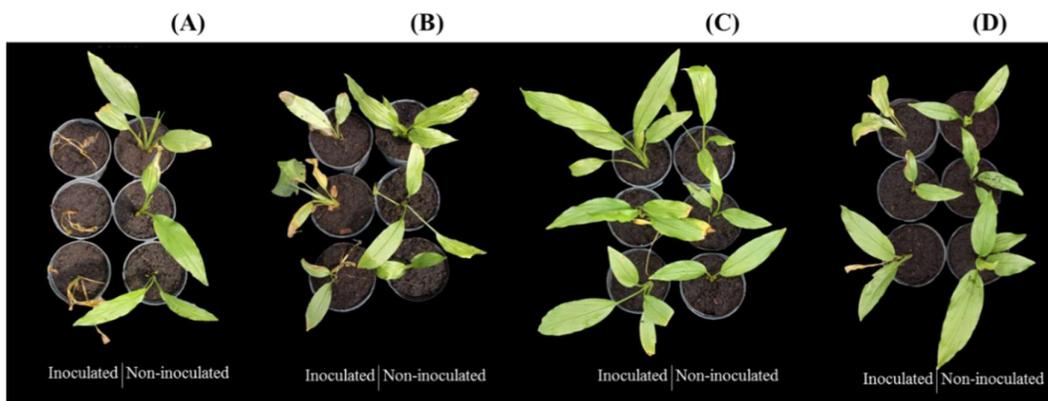


Figure 4 (A) Wild-type turmeric and electron-beam-irradiated populations: (B) EBRTP-2, (C) EBRTP-4 and (D) EBRTP-6. The pictures were taken after the roots were dipped in an *R. solanacearum* suspension for 24 h and the plants were grown in pots containing soil and leaf mold (1:1 ratio) for 14 days.

Conclusions

This study provides information on electron-beam-induced mutations of turmeric. The optimal electron beam dose for an *in vitro* turmeric culture was determined. Three irradiated M_1V_5 populations (EBRTP-2, EBRTP-4 and EBRTP-6) showing resistance to bacterial wilt disease were isolated. However, a more detailed study involving a larger number of irradiated populations, different bacterial strains and selection techniques against bacterial wilt disease, as well as experiments on the field and yield trials are necessary to identify the turmeric genotypes that should be integrated into a breeding program focused on resistance to bacterial wilt disease.

Acknowledgements

This study was supported by research funds from the Graduate School of Kasetsart University, Bangkok, Thailand. We also would like to thank the Agricultural Research Development Agency (ARDA), Thailand for the research grant. Special thanks to the Thailand Institute of Nuclear Technology (Public Organization) for their assistance with electron beam irradiation and Trang Horticultural Research Center, Department of Agriculture for supporting turmeric rhizome. The authors deeply appreciate the Nuclear Technology Research Center (NTRC) for the use of its equipment and facility support.

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