



# Productivity of L-DOPA in *in vitro* shoots of *Mucuna pruriens* var. *utilis* enhanced by gamma radiation

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## ABSTRACT

This research aimed to study the ability to produce levodopa (L-DOPA) in an *in vitro* shoot culture of *Mucuna pruriens* var. *utilis* developed from gamma-irradiated seeds, to be used as an alternative method for producing L-DOPA. A ultraperformance liquid chromatography system was used for L-DOPA quantification. The method was validated and it revealed good sensitivity, specificity, precision, and accuracy. Irradiation of the seeds at a 200–500 Gy gamma dose exhibited the median lethal dose at 336.75 Gy. The shoots developed from seeds germinated after irradiation with 200 and 300 Gy gamma dose produced L-DOPA at levels 300% and 220% higher than the nonirradiated group, respectively, whereas the shoot formation was unsuccessful when the radiation increased to 400 and 500 Gy. The results demonstrated that irradiation of the seeds at 200 and 300 Gy gamma can be used to produce *in vitro* shoots for L-DOPA production.

## INTRODUCTION

L-DOPA (known as levodopa and 3,4-dihydroxyphenyl-L-alanine) is a metabolite with diverse biological functions in humans and some plants. In humans, it is a precursor of dopamine which is a brain neurotransmitter and can penetrate the blood-brain/blood-cerebrospinal fluid barrier. A deficiency of dopamine in the brain can result in motor function impairment, which is a major cause of physical disability in patients with Parkinson's disease (PD) (Jankovic, 2008). Since L-DOPA can increase the dopamine level in the brain, the administration of L-DOPA is commonly recommended for treating motor symptoms of PD (Nagatsu and Sawada, 2009). The current supply of L-DOPA for drug manufacturing is produced by the chemical synthetic process (Knowles, 2004). Nevertheless, the synthetic process for L-DOPA production is complex and produces low yield and low enantioselectivity (Min *et al.*, 2015). Alternative approaches, such as biotransformation and microbial fermentation, have been

utilized to improve the production yield and the enantioselectivity. Nevertheless, employment for industrial-scale production has been limited by low production capacity due to the complex purification process involved (Min *et al.*, 2015).

It has been well established that *Mucuna* sp. (family Leguminosae) is rich in L-DOPA. Plant-derived L-DOPA is enantioselective, and the purification of L-DOPA from plants does not require a complicated process. It has been observed that *Mucuna pruriens* var. *utilis* contains 3.1%–6.1% L-DOPA in the seeds (Kavitha and Thangamani, 2014). The concentrations are high enough for commercial extraction. However, there are some limitations to producing L-DOPA from seeds. For instance, the pod has strident trichomes that cause a very strong itching sensation, and the success of cultivation greatly depends on the environmental conditions (Ingle, 2003). Therefore, *in vitro* plant cultivation has been an interesting alternative source for L-DOPA production because it takes a shorter time, and it is easier to control the environment during production. The formation of L-DOPA has been found in callus cultures (Brain, 1976), suspension cultures (Wichers *et al.*, 1993), and regenerated plants (Lahiri *et al.*, 2011) of *M. pruriens* var. *utilis*. However, the concentrations of L-DOPA were less than those accumulated in the seeds.

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Gamma rays are often employed to enhance the amounts of pharmacologically important metabolites. Gamma irradiation can be applied to plants or plant parts, as well as *in vitro* cultures (Vivek and Shukla, 2017). For example, irradiation of *Psoralea corylifolia* seeds at 20 kGy resulted in an increase in the psoralen content as high as 32-fold. The callus cultures of *Stevia rebaudiana* produced higher stevioside content after irradiation with 15 Gy. Irradiation of *Panax ginseng* hairy root cultures at 100 Gy stimulated saponin and ginsenosides contents as high as 1.4-fold and 1.8-fold, respectively. In addition, the camptothecin yield in the callus tissue of *Nothapodytes foetida* was also reported to increase by 20-fold with 20 Gy. Several studies have stated that gamma irradiation increased plant metabolite production by affecting the activity of secondary metabolite biosynthetic enzymes.

Gamma irradiation has also been applied to induce mutation in seeds of *M. pruriens* var. *utilis* for establishing plants producing a high level of L-DOPA (Singh *et al.*, 2016). In the referenced study, gamma-ray irradiation at doses of 50–500 Gy was applied to the seeds and the mutant plants that provided high L-DOPA contents in the seed were selected. The mutant that produced the highest L-DOPA content could produce L-DOPA as twice as high as the parent plant. The study also revealed the suppression of the dopa-decarboxylase gene, which is responsible for the conversion of L-DOPA to dopamine, in the mutants with a high level of L-DOPA.

This research aimed to study the ability to produce L-DOPA in shoots of *M. pruriens* var. *utilis* cultivated *in vitro* from gamma-irradiated seeds, to be used as an alternative method for producing L-DOPA. In this study, a validated ultraperformance liquid chromatography (UPLC) method was used to determine the amount of L-DOPA in shoots (Wachisunthon *et al.*, 2019).

## MATERIALS AND METHODS

### Plant material

The seeds of *M. pruriens* var. *utilis* Wall. ex Wight were obtained from plants grown in a greenhouse of the Medicinal Plant Research Institute, Department of Medical Sciences, Ministry of Public Health, Nonthaburi, Thailand.

### Gamma irradiation and shoot production

Gamma irradiation was conducted at the Thailand Institute of Nuclear Technology at Naknonayok, Thailand, using a cobalt-60 source at a dosage rate of 115 Gy/hours<sup>-1</sup>. Gamma irradiation was carried out at 0, 200, 300, and 400 Gy (30 seeds each). The total radiation dose absorbed by seeds was further confirmed by FWT50 alanine pellet dosimeters (Far West Technology, Inc., Goleta, CA). After irradiation, *in vitro* cultivated plants were prepared by adapting the method of Sathyanarayana *et al.* (2008). The seeds were surface-sterilized and germinated on Murashige and Skoog (MS) basal medium (Murashige and Skoog, 1962) at 25°C ± 2°C with a 16-hours light/8-hours dark photoperiod. On day 14, the number of sprouted seeds was recorded to determine the median lethal dose (LD<sub>50</sub>). After germination, the nodal parts were used as explants and inoculated onto the MS medium supplemented with 2 µM 6-benzylaminopurine (BAP). The cultures were incubated at 25°C ± 2°C under a 16-hours photoperiod with a cool white fluorescent light for 30 days.

### Extraction of L-DOPA from *in vitro* cultivated plants

The stems and the leaves of *in vitro* cultivated plants were dried using a hot air oven at 50°C. The dried plants were ground to a fine powder with a granite mortar and pestle. The plants were extracted by adapting the method of Hasegawa *et al.* (2011). Ten milligrams of dried sample was mixed with 500 µl of acetonitrile/water/formic acid (50 : 50 : 1). The mixture was extracted using an ultrasound-assisted extraction method by sonication in an ultrasonic bath for 20 minutes at room temperature. Then, it was centrifuged at 10,000 rpm for 5 minutes. The supernatant was collected, and the precipitates were reextracted under the same conditions. After the second extraction, the precipitates were washed three times with 300 µl of acetonitrile/water/formic acid (50 : 50 : 1). All the supernatants were combined and produced up to 2 ml using a volumetric flask.

### Sample preparation by solid-phase extraction (SPE)

The extract was cleaned by SPE using a Sep-Pak si (500 mg; 3 ml SPE cartridge). The extract was wetted with 3 ml of acetonitrile. Then, 100 µl of the extract was loaded on the packing bed of the cartridge. Next, it was washed with 2 ml of acetonitrile, and finally, L-DOPA was eluted from SPE with 4 ml of methanol/water/formic acid (96 : 3.5 : 0.5). This final fraction was evaporated to a dry state using a nitrogen evaporator. The residue was dissolved with 200 µl of acetonitrile/water/formic acid (50 : 50 : 1) and filtered through a 0.2 µm membrane filter before analysis by ultraperformance liquid chromatography (UPLC).

### Preparation of the standard solution

A stock standard solution of 1 mg ml<sup>-1</sup> was prepared by dissolving 2 mg of standard L-DOPA (Sigma-Aldrich, St. Louis, MO) in 2 ml of acetonitrile/water/formic acid (50 : 50 : 1). Working solutions with 5–100 µg ml<sup>-1</sup> were prepared by diluting the stock solution with the same solvent.

### Chromatographic condition

The analysis was carried out using an ultraperformance liquid chromatography (UPLC) system (Waters, Milford, MA) with a photodiode array detector. The chromatographic condition was developed and recently published elsewhere (Wachisunthon *et al.*, 2019), i.e., the sample was separated on a Luna Omega Polar C18 (150 × 2.1 mm, 1.6 µm) column (Phenomenex, Milford, MA). The column temperature was 35°C. The mobile phase was a mixture of 0.1% formic acid in water and methanol with a ratio of 94.6 : 5.4 at a flow rate of 0.25 ml/minutes<sup>-1</sup>. The injection volume was 1 µl, and the detection wavelength was 280 nm. Method validation was carried out according to the ICH guidelines (ICH Expert Working Group, 2005).

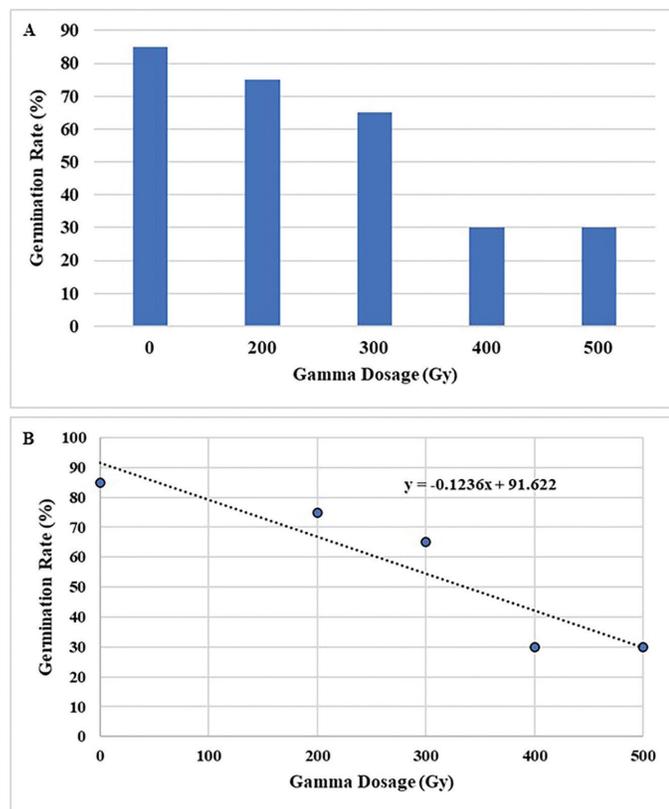
## RESULTS AND DISCUSSION

Gamma ray is an electromagnetic radiation that influences physiological and biochemical processes in plant cells and tissues, leading to the modification of plant phenotype and genotype (Sumira *et al.*, 2012). These modifications rely on the intensity of radiation energy absorbed by plant tissues (Marcu *et al.*, 2013). In this study, it is apparent that a high dose of gamma rays could hamper seed germination. Within a 14-days incubation period, the germination percentage observed from the

nonirradiated seeds was 85%. The germination rate diminished to 65%–75% when the seeds were treated with a 200–300 Gy gamma dose and decreased to less than 40% when they were treated with a 400–500 Gy gamma dose (Fig. 1A). The median LD<sub>50</sub> determined based on the intersection of the 50% germination line with the germination curve was at 336.75 Gy (Fig. 1B). The appearance of the *in vitro* shoots is shown in Figure 2.

Gamma irradiation also affected subsequent shoot growth. The number of shoots was reduced in the groups exposed to the higher doses (400 and 500 Gy) of gamma radiation compared to the control group (Table 1), and the shoots became deformed with yellow spots and were unable to survive. The results agreed with the radiation sensitivity testing reported previously, which showed that a low dose of gamma radiation was more effective for promoting seed germination and plant growth. For example, *Lathyrus chrysanthus* seed germination and shoot initiation were achieved by irradiation of seeds at 100–150 Gy gamma dose but were unsuccessful when the radiation increased to 200 Gy (Beyaz *et al.*, 2016). It has also been reported for cucumber and okra seeds that greater numbers of seeds germinated after irradiation with a 50 Gy gamma dose compared to that with 100 and 200 Gy gamma doses (Norfadzrin *et al.*, 2017). The ability of seed germination was diminished after exposure to higher doses of radiation, which may have been associated with chromosomal impairment and subsequent cell division retardation (Neelam *et al.*, 2014).

This study also revealed that irradiation of *M. pruriens* var. *utilis* seeds with 200 and 300 Gy gamma doses could induce

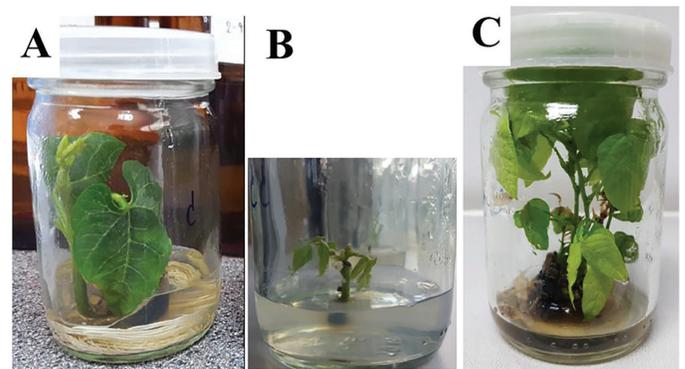


**Figure 1.** Germination percentages of gamma-irradiated seeds (A) and radiation survival curves (B) for *M. pruriens* var. *utilis* seeds.

the accumulation of L-DOPA. The multiple shoots from the 200 and 300 Gy gamma doses were maintained for 30 days before they were harvested and measured for L-DOPA content and were found to contain L-DOPA at approximately two times the level of the nonirradiated group (Table 2 and Fig. 3). The yield of L-DOPA was greater for field-cultivated seeds (3%–6%) than for *in vitro* shoots (1.4%); however, the period from flowering to mature seed took 2–3 months, and the plants died 45–60 days after producing a seed (Fact Sheet - *Mucuna pruriens* - tropical Forages, 2018; Kavitha and Thangamani, 2014). Thus, the growing shoots *in vitro* were an appealing choice for L-DOPA production. In addition, the biomass amounts obtained from the 200 and 300 Gy gamma irradiation groups were 1.8 and 1.4 times higher than the nonirradiated group, respectively (Table 2). Taken together, considering the L-DOPA content and the biomass, shoots growing from seeds irradiated with 200 and 300 Gy gamma doses produced L-DOPA at levels of 300% and 220% higher than the nonirradiated group, respectively (Fig. 4).

The application of gamma radiation to enhance secondary metabolite accumulation or to induce synthesis has been supported by various authors (Ahamed, 2019; Akshatha *et al.*, 2013; Fulzele *et al.*, 2015; Kovacs and Keresztes, 2002). The gamma ray is known to be an abiotic elicitor that promotes the intracellular synthesis of oxygen radicals and hydrogen peroxide. These radicals may cause injury to plant cells and can affect certain important metabolic functions that might be crucial for plant survival (Wi *et al.*, 2007). Production of L-DOPA might involve a defense system that responds to stress induced by gamma radiation.

In this study, L-DOPA was quantified using a UPLC method that was developed and validated for the separation and determination of L-DOPA in plantlets of *M. pruriens* var. *utilis* (Wachisunthon *et al.*, 2019). Since plant leaves accumulate various metabolites that could impair the efficiency of analysis, SPE was applied to purify the sample before UPLC was performed. The method was suggested to be specific because the retention time (2.029 minutes) of the sample was equal to that of the L-DOPA standard, and there was no interference from the blank at the retention time of the standard. The calibration curve was linear



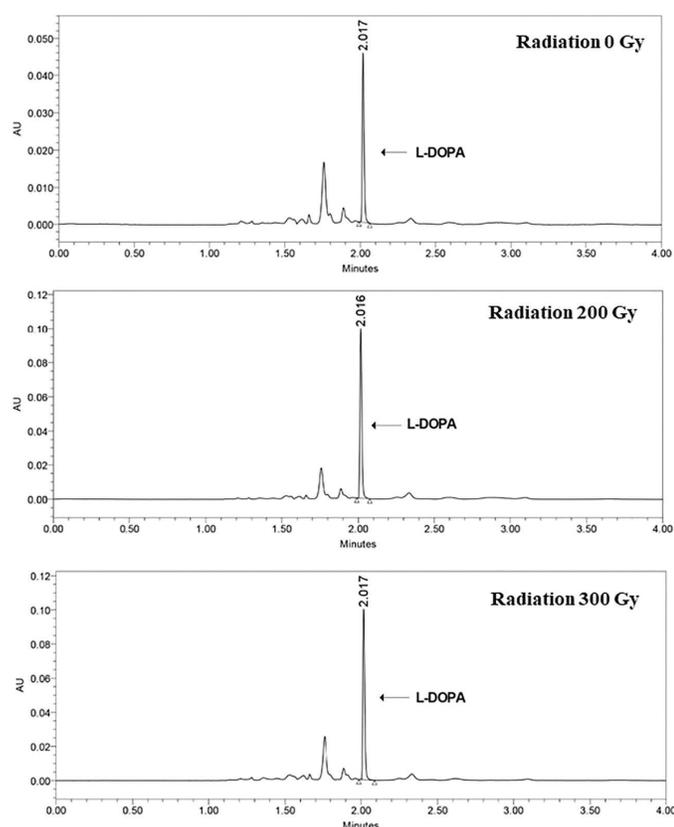
**Figure 2.** *Mucuna pruriens* var. *utilis* plantlets after seed germination (A), *in vitro* shoots initiated from a nodal explant cultured in the MS medium supplemented with 2  $\mu$ M BAP at 25°C  $\pm$  2°C under a 16-hours photoperiod with cool white fluorescent light (B), and *in vitro* shoots cultured for 30 days (C). The pictures are from the 200 Gy gamma dose.

**Table 1.** Number of shoots and their appearance.

Gamma radiation (Gy)	Number of shoots per explant (mean $\pm$ SD)	Appearance
0	3 $\pm$ 0.8	Green healthy leaves
200	3 $\pm$ 1.5	Green healthy leaves
300	2 $\pm$ 0.8	Green healthy leaves
400	0.8 $\pm$ 0.3	The leaves were puckering and the colour was yellow. The plants die within 2 weeks.
500	1 $\pm$ 0.5	The leaves were puckering and the colour was yellow. The plants die within 2 weeks.

**Table 2.** Biomass and L-DOPA content obtained from shoots after a 30-days incubation period.

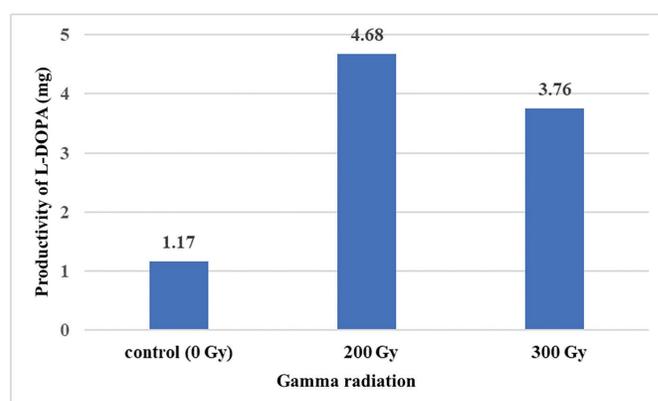
Radiation (Gy)	Fresh weight (g)	Dry weight (mg)	L-dopa content* (%w/w) (%RSD, $n = 3$ )
0	1.81	175.23	0.67 (1.38)
200	3.20	325.22	1.44 (0.89)
300	2.45	252.55	1.49 (1.58)

**Figure 3.** Chromatograms of L-DOPA derived from shoots after a 30-days incubation period.

at the concentration range of 5–125  $\mu\text{g ml}^{-1}$  of L-DOPA, and the correlation coefficient (R) was 0.9997. The limit of detection and limit of quantitation were 3  $\mu\text{g ml}^{-1}$  and 5  $\mu\text{g ml}^{-1}$ , respectively. The precision studies revealed that the percent relative standard deviations (%RSDs) of the L-DOPA content for repeatability and intermediate precision were 1.89 and 2.46, respectively. Finally, the recovery of L-DOPA from spiked samples was 89%–92%.

## CONCLUSION

Acute gamma irradiation of *M. pruriens* var. *utilis* seeds at the doses of 200 and 300 Gy has been proven to enhance L-DOPA production from *M. pruriens* var. *utilis* shoot cultures.

**Figure 4.** Productivity (calculated based on dried weight) of L-DOPA derived from shoots after a 30-days incubation period.

However, further studies should be carried out to produce a high-yielding clone, as well as maintain genetic stability, to establish genetically stable shoot cultures as an alternative L-DOPA source.

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## AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work.

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There is no funding to report.

## CONFLICTS OF INTEREST

The authors report no conflicts of interest in this work.

## ETHICAL APPROVAL

This study does not involve the use of animals or human subjects.

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