Chapter

Gamma Irradiation Causes Variation and Stability of Artemisinin Content in *Artemisia annua* Plants

Thongchai Koobkokkruad, Praderm Wanichananan, Chalermpol Kirdmanee and Wanchai De-Eknamkul

Abstract

Artemisinin is an anti-malarial sesquiterpene lactone isolated from *Artemisia annua* L., a traditional Chinese herb of the family Asteraceae. The plant contains relatively low artemisinin content, ranging from 0.01 to 0.8% of the plant dry weight, depending on the geographical origin, seasonal, and somatic variations. Ionizing radiation has been recognized as a powerful technique for plant improvement, especially in crop plants. This technique creates genetic variability in plants, which can be screened for desirable characteristics. Very little is known about the effect of gamma irradiation on the potential increase of artemisinin production in *A. annua*. In this study, 130 shoot tips excised from the population of in vitro *A. annua* plantlets (with an average leaf artemisinin content of 0.18 ± 0.09%) were exposed to 5 Gy ^{60}Co gamma irradiation and subsequently transferred to a suitable medium for in vitro development of plantlets. The resulting 90 stable survived after four passages appeared to have a wide variation of artemisinin content, ranging from 0.02 to 0.68% of dry weight. All the viable plantlets were then transferred from the in vitro cultures to ex vitro conditions both in a greenhouse and an open field. A significant correlation was observed between artemisinin content among individual pairs of the vitro plantlets and ex vitro mature plants, with the correlation coefficient (R^2) values of 0.915 for the greenhouse plants and 0.797 for the open field plants. Among these, the highest artemisinin-containing plant appeared to accumulate 0.84% artemisinin of dry weight in the open field, which is almost five times higher than the original plants. These results suggest that gamma irradiation with 5-Gy dose can produce viable variants of *A. annua* that can maintain the biosynthetic capability of artemisinin throughout the in vitro-ex vitro transfer and development of the first generation of mature plants.

Keywords: artemisinin, *Artemisia annua* L., gamma irradiation, in vitro plantlets, ex vitro plants

1. Introduction

Artemisinin is a natural sesquiterpene lactone containing an unusual peroxide bridge (**Figure 1**) [1]. It is present mainly in the leaves of *Artemisia annua L.* (family Asteraceae) by storing in the glandular trichomes, which are tiny specialized hair-like epidermal cells found on the epidermis of leaves [2]. This traditional Chinese herb is a wild growing species with relatively low artemisinin content, ranging from 0.01 to over 1% of the plant dry weight, depending on the geographical origin, seasonal, and somatic variations [3, 4] and density of glandular trichomes in the leaves and aerial parts [5]. At present, the only commercial source of artemisinin is by extraction from field-grown leaves and flowering tops of the plant although many attempts to obtain higher artemisinin yield have been made from using simple breeding programs to complicated biotechnological approaches (for review, see [6]). Total synthesis of the compound has been reported [7, 8], but many chemical steps are required and the yields are low. In vitro cultures of *A. annua*, such as cell suspension and callus [9], shoot [10, 11] and hairy root cultures [12–15], have also been established for studying their potentials of producing artemisinin, but in vitro culture for artemisinin production has yet to prove commercially feasible. Therefore, the whole plant of *A. annua* is still the most economic source of artemisinin, and the development of high-producing plants of *A. annua* seems to be the main direction to obtain large quantities of relatively cheap artemisinin.

Ionizing radiation has been recognized as a powerful technique for plant improvement, especially in crop plants [16–18] and medicinal plants (for review, see [19]). This technique creates genetic variability in plants, which can be screened for desirable characteristics. So far, very little is known about the effect of gamma irradiation on the potential of artemisinin biosynthesis in *A. annua*, which involves several steps in its pathway [6]. Previously, we have reported a method for establishing in vitro plantlet variants of *A. annua* using low-dose gamma irradiation (less than 10 Gray) [20]. The survived plantlet variants maintained under the in vitro conditions for more than 6 months appeared to have stable content of artemisinin. However, it remained unknown whether the changes in artemisinin biosynthesis in in vitro plantlets would be maintained when the plants are grown ex vitro in a greenhouse or an open field. This question prompted us to investigate the process of acclimatization of the plantlets, followed by evaluation of the artemisinin content in the resulting whole plants.

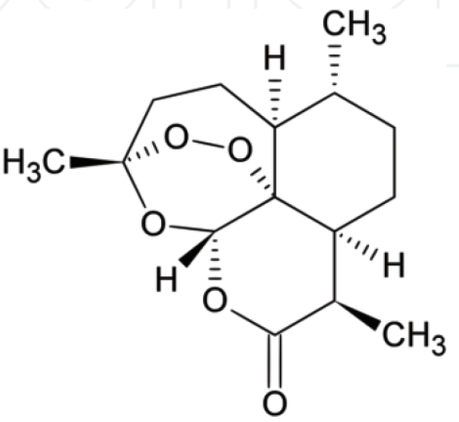

Figure 1.
The structure of artemisinin [1].

2. Application of the gamma irradiation technique for potential increase of artemisinin accumulation in *A. annua*

2.1 Effects of gamma irradiation on the morphology and survival of *A. annua* plantlets

In this study, mature seeds of *A. annua* were first surface sterilized and germinated on the MS medium, supplemented with 3% sucrose and solidified with 0.8% agar. The cultures were incubated for 2 months at 25°C with an exposure to 16 h light (ca. 3000 lux) and 8 h dark cycle. The obtained plantlets were then subcultured for five times before their shoot tips were excised and treated with gamma radiation. Practically, 1000 shoot tips (ca. 5 mm) excised from the in vitro plantlets were placed onto the same MS medium and irradiated with gamma rays generated by Cobalt-60 at the dose rate of 8.56 Gy min^{-1} (using the facilities at the Office of Atomic Energy for Peace (OAEP), Bangkok, Thailand). With this dose rate, the amount of irradiation energy absorbed by the shoot tips from 1 to 10 Gray (Gy) was conducted using the irradiation times from 7 to 70 s. After the irradiation, the exposed shoots were transferred to the fresh hormone-free MS medium. The shoots with subsequent active growth were subcultured every six weeks for four times on the same hormone-free MS medium. All the cultures were grown under the same conditions. After the forth passage, each vigorous shoot was cultured on the hormone-free MS medium in a 230-ml glass bottle. After culturing for six weeks, survival percentage and regrowth ability were recorded.

Figure 2 shows the morphology of *A. annua* plantlets derived from shoot tips gamma irradiated with a low dose range from 1 to 10 Gy. The plantlets depicted are representative of populations irradiated with the indicated gamma ray doses that survived four subsequent passages over a period of more than 6 months. The lowest dose of 3 Gy appeared to promote the growth of the plantlets, whereas doses above 5 Gy led to significant growth and morphological abnormalities. As shown in **Figure 2**, the 8-Gy dose gave rise to plantlets with pale green, fully expanded leaves, and the 10-Gy dose resulted in dwarf plantlets with no root differentiation.

In terms of survival rate, the results showed that there was a continual reduction in the survival percentage of the in vitro plantlets with increase in gamma

Figure 2.
Effects of low dose of gamma irradiation on the morphology of A. annua plantlets.

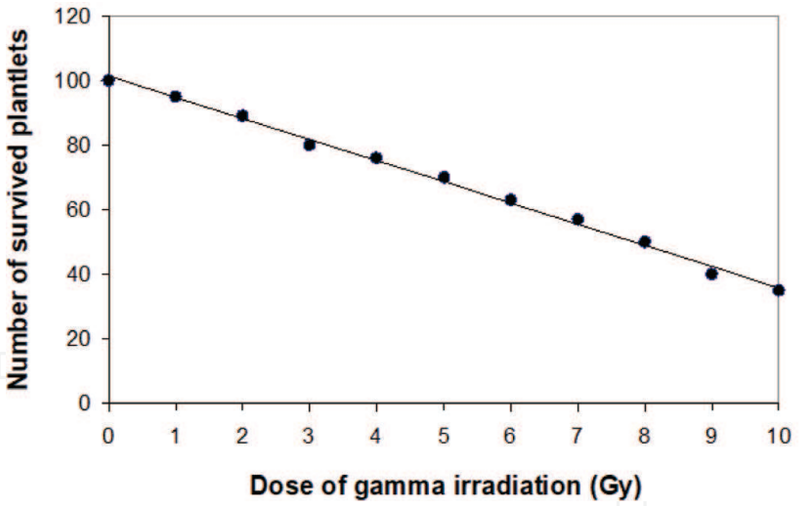

Figure 3.
The effect of various doses of gamma irradiation on the survival of in vitro plantlets of A. annua.

irradiation dosage from 1 to 10 Gy (**Figure 3**). The lethal dose of gamma rays that causes 50% survival reduction (LD_{50}) was 8 Gy. Again, the doses lower than this LD_{50} value showed essentially normal morphology of the survived plantlets, whereas the higher doses seemed to cause significant abnormalities as shown by dwarf plantlets with pale leaves (**Figure 2**). This LD_{50} value was obtained from the in vitro plantlets survived for at least 6 months (four subsequent subcultures) after the irradiation. It is, therefore, likely that they are genetically stable variants.

2.2 Effects of gamma irradiation on artemisinin accumulation in *A. annua* in vitro plantlets

To perform a rapid analysis of artemisinin in a large population of the irradiated plantlets, we used our own developed simple and sensitive TLC-densitometric method for artemisinin analysis which was reported previously [21]. Practically, fresh leaves obtained from various in vitro plantlets were collected, dried at 60°C, and ground to fine powder in a grinder. Each powder sample (100 mg) was extracted under reflux in 10 ml hexane (70°C) for 1 h. The extract was then filtered, and a 10 μl aliquot was spotted onto a pre-coated silica gel TLC plate. Up to 15 samples could be applied onto each plate which was developed using the solvent system of hexane:ethyl acetate:acetone, 16:1:1. The plate was dried and exposed for 2 h with saturated ammonia vapor (in a closed TLC tank) for complete derivatization of artemisinin. The TLC plate was then taken from the tank, air dried, and observed in a light box under the wavelength of 366 nm which could be seen variation of artemisinin band intensity among various extract samples (**Figure 4A**). The plate was then scanned by a TLC densitometer under the wavelength of 320 nm to obtain corresponding TLC-densitometric chromatograms. The area under artemisinin peak of each sample was then converted to artemisinin content based on a calibration curve that showed linearity from 0.06 to 12 μg ml^{-1} of artemisinin.

Using this TLC-based technique, the surviving plantlets (obtained after 8 Gy treatment and four subsequent subcultures) were analyzed for their ability to accumulate artemisinin. The results showed a wide variation of artemisinin content, ranging from 0.03 to 0.70% dry weight (**Figure 5**). The control plantlets derived from the shoot tips not exposed to gamma rays showed their artemisinin levels of as low as 0.18 ± 0.09% of dry weight. In terms of content distribution, almost 80% of the irradiated plantlets showed artemisinin content less than 0.3%,

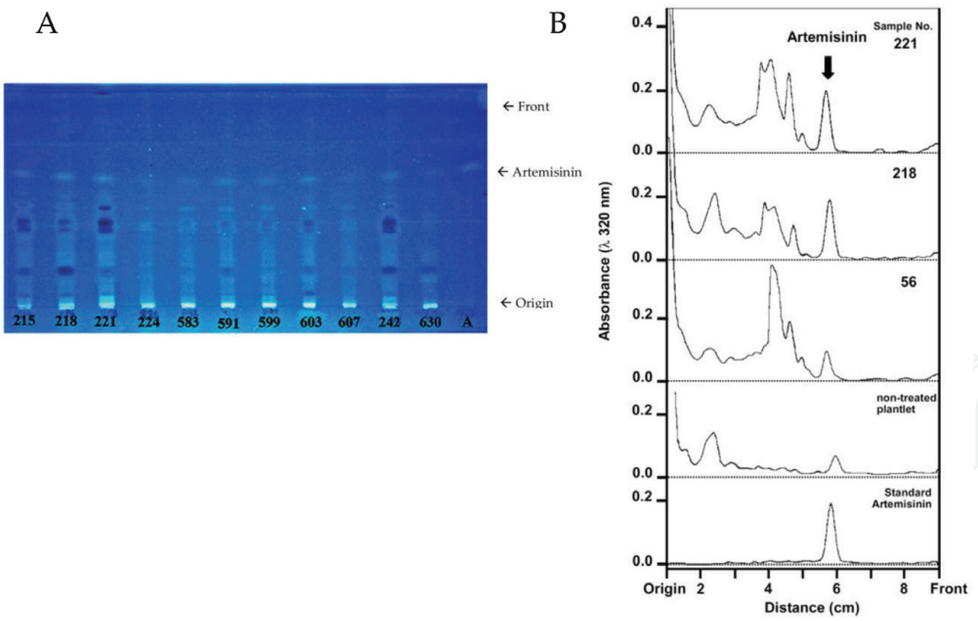

Figure 4.
(A) Typical TLC-patterns of some A. annua crude extracts prepared from the population of gamma-irradiated plantlets. Variation of artemisinin band intensity among the samples can be observed under 360 nm after exposed with ammonia at 100°C for 2 h. (B) TLC-densitometric chromatograms obtained by scanning at 320 nm of some samples of the TLC plate shown in (A).

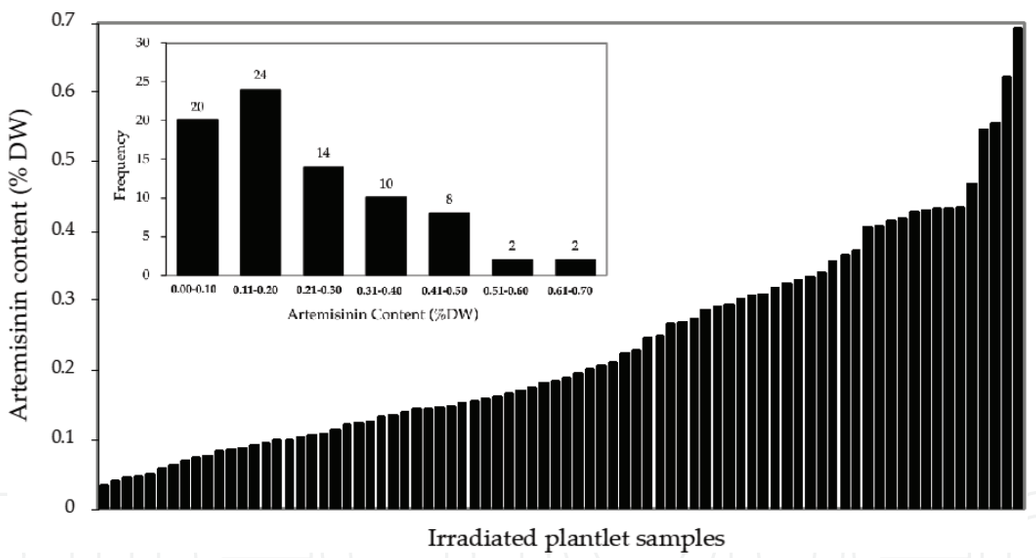

Figure 5.
Variation of artemisinin content in various plantlets of A. annua that their shoot tips had been exposed to a dose of 8 Gy of gamma rays.

and approximately 5% of the population showed higher than 0.5% of dry weight (**Figure 5** inset). Thus, it was clear that there were variations in the potential of artemisinin biosynthesis among the irradiated plantlet population.

It should be noted that more than 50% of the plantlet population accumulates artemisinin in the content higher than the original untreated plants (0.18 + 0.09% of dry weight). This is probably due to the use of low-artemisinin containing plants as a starting material, which allow higher artemisinin-containing variants be obtained more easily upon the irradiation. The observed quantitative and some extent of qualitative variations indicate that the secondary metabolism in the irradiated plantlets of *A. annua* is affected considerably by the treatment of the low-dose gamma irradiation.

2.3 Correlation between artemisinin accumulation and enzyme activity of amorpha-4,11-diene synthase in irradiated plantlets

The observed variation of artemisinin accumulation in the irradiated plantlets raised a question on the possible site genes of mutation, especially of the genes involved in the biosynthetic pathway of artemisinin. Since amorpha-4,11-diene synthase (ADS) has been known as one of the key enzymes of the pathway in *A. annua* [22], it was selected as a target for studying the relationship between its enzyme activity and artemisinin accumulation. In this study, a crude enzyme extract of each plantlet sample was prepared by quick freezing the fresh leaves (5 g) in liquid nitrogen and ground in a pre-cooled mortar. The resulting fine powder was added with 15 ml cold extraction buffer containing 5 mM 3-(N-morpholino) propanesulfonic acid (Mops) buffer, pH 7.0, 10% (v/v) glycerol, 1 mM ascorbic acid, 10 mM $MgCl_2$, and 2 mM dithiothreitol (DTT). After 15 min of stirring, the suspension was passed through four layers of cheesecloth, and the filtrate was centrifuged 100,000 × *g* at 4°C. The supernatant was then desalted by passing through a PD-10 column. The filtrate was used as crude enzyme extract and kept at −80°C before being used for determining the enzyme activity of ADS.

The enzyme activity of ADS was then determined by modifying the radioisotopic method described previously [22]. The reaction mixture contained [1-^{3}H(N)] farnesyl diphosphate (100,000 dpm), 5 mM Mops buffer, pH 7.0, 10%(v/v) glycerol, 10 mM ascorbic acid, 10 mM $MgCl_2$, 2 mM DTT, and 10 mM Na_2MoO_4 in a total volume of 70 μl. After 30 min of incubation at 30°C, the reaction mixture was extracted with 1 ml hexane, taking the hexane layer to evaporate followed by spotting onto a TLC plate (aluminum sheet, silica gel 60 F254, 0.25 mm thickness). The resulting TLC plate was then developed in a solvent system of hexane:ethylacetate:acetic acid (25:7:1) and was scanned to obtain radio-chromatograms by a TLC-radioscanner. The area under amorpha-4,11-diene peak of each radio-chromatogram was then used for calculating the synthase activity. **Figure 6** shows typical TLC-radiochromatograms of the reaction mixtures catalyzed by enzyme preparations obtained from some irradiated plantlets. It can be seen that the radioactive peaks of amorpha-4,11-diene (Rf value of 0.55) could be clearly detected with different peak sizes from different samples, suggesting that the TLC-radio assay worked well for determining the enzyme activity of ADS.

Subsequently, 18 plantlet samples with different artemisinin contents were assayed for their ADS activity. Again, it was found that the selected samples showed high variation in the enzyme activity, ranging from 0.02 to 0.18 pkat mg^{-1} protein. When the results of the enzyme activity and artemisinin content were plotted together (**Figure 7**), it was found that the 18 plantlets showed their poor value of the correlation coefficient at R = 0.300 (**Figure 7A**). However, by excluding only two outliners with extremely high ADS activities, the correlation coefficient appeared to be much better, with R = 0.717 (**Figure 7B**). Among these, 11 of the 16 samples showed quite high value of the correlation coefficient, R = 0.922 (**Figure 7C**). This suggested that the gamma irradiation did affect the gene of ADS, and thus the biosynthetic capability of artemisinin in the mutant plants.

2.4 Variations in artemisinin content in plantlets irradiated with the 5-Gy dose

As mentioned earlier (Section 2.1), the doses above 5 Gy led to significant growth and morphological abnormalities, with the 8-Gy dose giving rise to

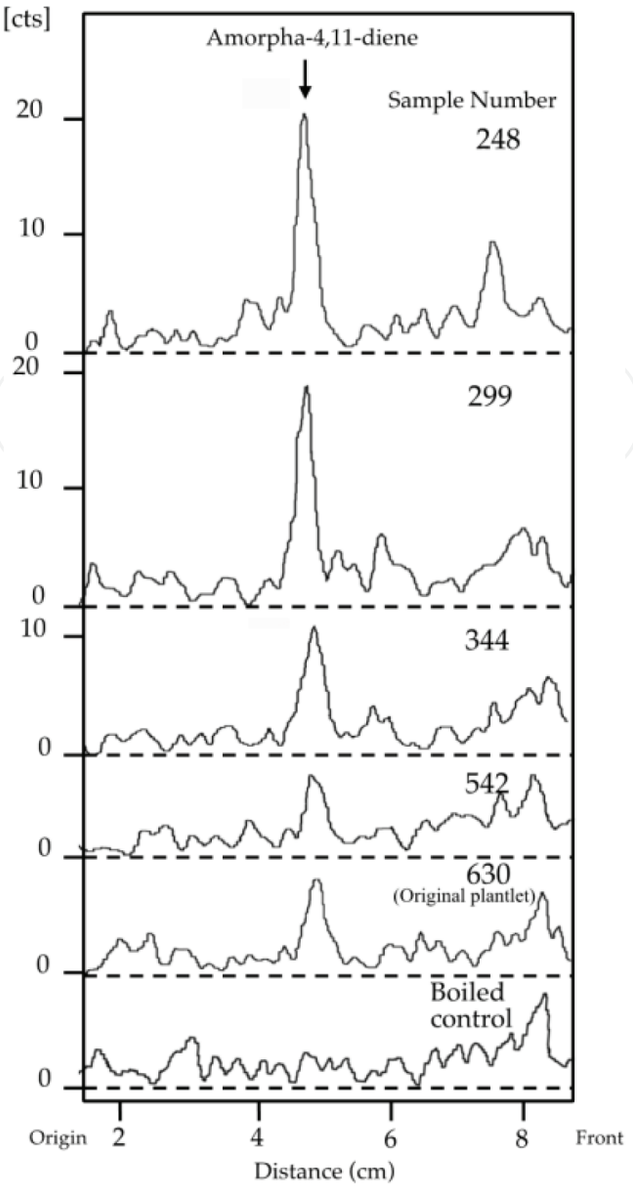

Figure 6.
Typical TLC-radiochromatograms obtained from a radio-based enzyme assay of ADS of some irradiated plantlet samples (indicated by the sample number) of A. annua. The reaction mixture of each ADS enzyme-assay sample was extracted with hexane followed by separation and detection of ADS product by a TLC-radioscanner.

pale-green and fully expanded leave plantlets, and the 10-Gy dose showing dwarf and no differentiate root plantlets with no root differentiation. Therefore, the 5-Gy dose was chosen for mass irradiation of shoot tips to obtain a population of *A. annua* plantlet variants to characterize the stability of changes in artemisinin biosynthesis in mature plants.

The results showed that among 130 shoot tips irradiated with a 5-Gy dose, 90 plantlets (69% survival) were obtained using established in vitro culture conditions. These plantlets were then evaluated for their ability of artemisinin accumulation. The results revealed again wide variation in artemisinin content, ranging from 0.02 to 0.68% dry weight (**Figure 8**). Among these plantlets, 6 individuals had an artemisinin content greater than 0.5% dry weight, 39 plantlets had an artemisinin content in the range of 0.21–0.50%, and 45 plantlets had an artemisinin content below 0.02%. As control plantlets derived from shoot tips not exposed to gamma rays exhibited an average artemisinin content of 0.18 + 0.09%, treatment with the

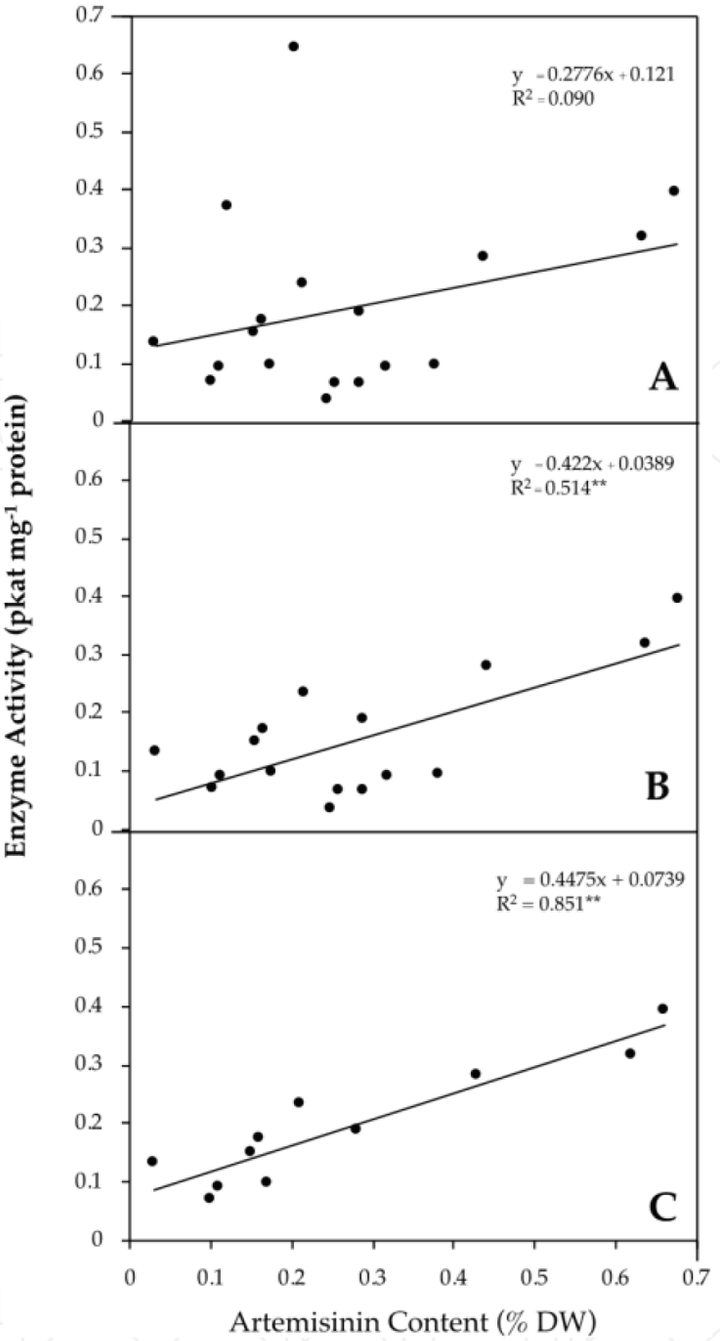

Figure 7.
Relationship between artemisinin content and ADS activity of various plantlets cultured in vitro. (A) The content-activity plot among 18 plantlet samples shows a poor value of the correlation coefficient (R) of 0.300, (B) the plot among 16 samples shows a moderate value of the correlation coefficient, R = 0.717 (P = 0.001), and (C) the plot among 11 samples shows a good value of the correlation coefficient, R = 0.922 (P = 0.001).

5-Gy dose resulted in an artemisinin content above that of the control for approximately half of the irradiated plantlets (**Figure 8**).

2.5 Ex vitro acclimatization of *A. annua* irradiated plantlets

In this study, actively growing shoot tips (length, ca. 5 mm) were excised from in vitro plantlets and stripped of their leaves. The resulting shoot tips (with 130 tips) were then inserted vertically 2 mm in depth into the MS medium containing 3% sucrose and 0.8% agar. Induction of variation in *A. annua*

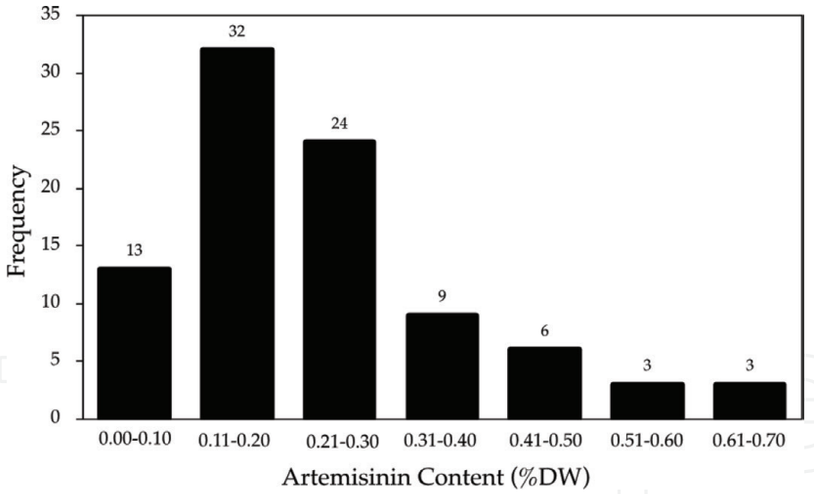

Figure 8.
Variations in artemisinin content in A. annua plantlets derived from shoot tips irradiated with 5 Gy of gamma rays.

plantlets using gamma irradiation (5 Gy) was then performed as described above (Section 2.1) and was transferred to the hormone-free MS medium for plantlet development. From each developed plantlet, selected shoots with two buds were excised, so that the upper bud would be regenerated to from shoots, and the lower bud regenerated to form roots. After four subculture passages, the leaves from healthy plantlets were harvested for artemisinin determination, and the shoots were used for a second round of plant regeneration. After 6 weeks, the in vitro plantlets were cleaned to remove the agar medium and were transferred into acclimatization conditions in 230-ml glass bottles. Each bottle contained 50 ml vermiculite and 50 ml MS medium without sucrose. The transferred plantlets were maintained at 25°C, with a photoperiod of 16 h of light at 40 μmol/m^2 s photosynthetic photon flux density provided by cool-white fluorescence lamps. After 8 weeks, a piece of membrane filter with a pore size of 0.5 μm was placed over a hole (area, 0.8 cm^2) in each plastic cap to increase air exchange in the culture vessel [23]. When the shoot of each plantlet grew sufficiently to fill the container, the plastic cap was loosened for one week and removed a week later to allow the shoot to continue growing out of the top of the bottle. The surviving plants (60 out of 90 plants) were then transferred from the vermiculite-based bottles to 10-cm pots containing sterile soil to grow for an additional 15 days under the same temperature and light conditions. Among the resulting 40 plants survived from the process of acclimatization, a group of 20 plants was transferred to a greenhouse at Chulalongkorn University, Bangkok, Thailand, and another group of 20 plants was transferred to an open field (30–38°C day air temperature and 20–29°C night air temperature) in Kanchanaburi Province, Thailand. These ex vitro plants were grown for a period of 6 months to reach maturity. At this point, leaves from the surviving plants (13 from the greenhouse group and 10 from the open field group) were harvested by random cuts of three different branches from each plant and prepared for determination of artemisinin content.

The development from the step of in vitro irradiated plantlets to ex vitro *A. annua* plants during the acclimatization process is shown in **Figure 9.** In general, both plant height and number of leaves increased continuously for plants grown in agar-MS (**Figure 9a**) and vermiculite-MS media (**Figure 9b** and **c**). In pots containing sterile soil, the plant height increased rapidly over 4 weeks, with the development

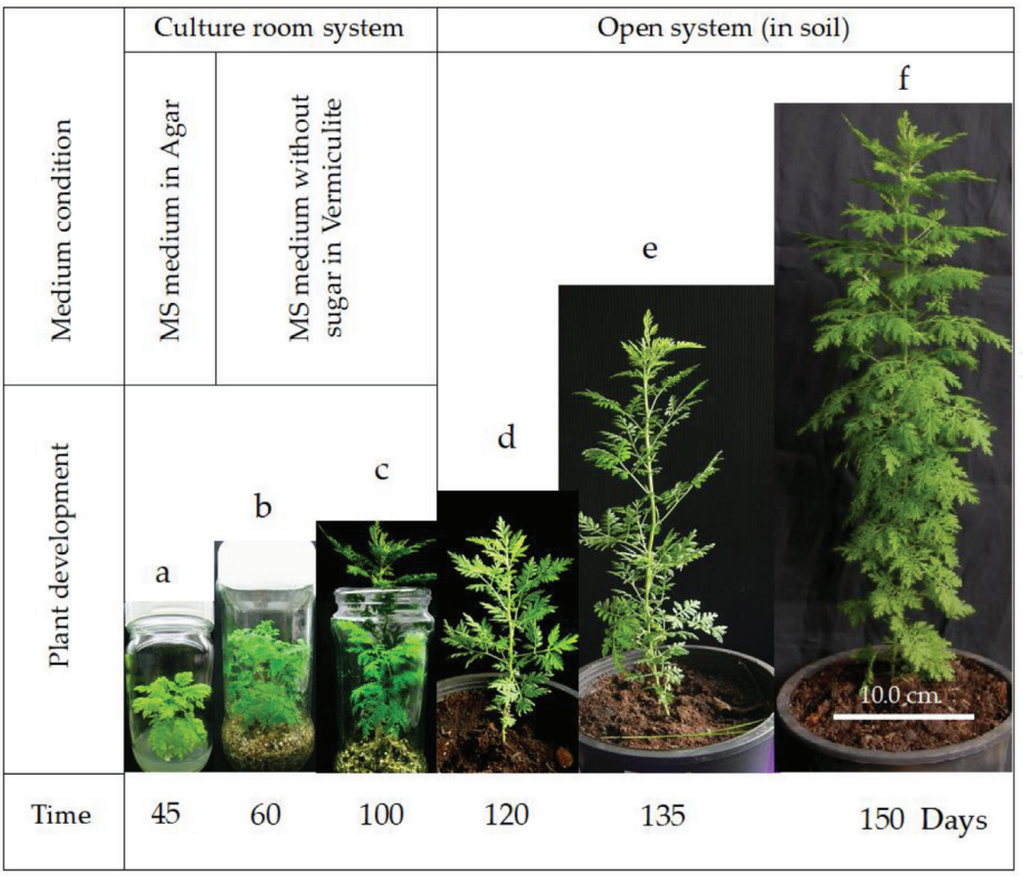

Figure 9.
Development of A. annua plants from in vitro plantlets to ex vitro plants. Six-week-old plantlets (a) were transferred to acclimatization conditions in 16-oz glass bottles containing 50 ml vermiculite and 50 ml MS medium without sucrose. After 8 weeks, (b) a piece of membrane filter (pore size: 0.5 μm) was placed over a hole (0.8 cm²) in each plastic cap to increase air exchange within the culture vessel. When the shoot of each plantlet completely filled the bottle, the plastic cap was loosened for one week and removed a week later to allow the shoot to grow above the top of the bottle (c). The surviving plants were then transferred from the vermiculite-based bottles to four-inch pots containing sterile soil for an additional 15 days of growth (d) prior to transfer to the greenhouse or an open field (135 days for (e) and 150 days for (f)).

of leaves similar to that of normal mature *A. annua* plants (**Figure 9d–f**). With respect to percent survival, the acclimatization protocol resulted in survival of 60 of the total 90 plantlets, corresponding to a survival rate of 66.7%.

2.6 One-to-one correlation of artemisinin content between in vitro plantlets and ex vitro plants

Quantitative analysis revealed that the 13 surviving plants from the in vitro-ex vitro transfer to the greenhouse had their artemisinin content ranging from 0.12 to 0.42% dry weight. This range was slightly narrower than that of the in vitro plantlets, which exhibited artemisinin contents ranging from 0.06 to 0.66%. Interestingly, comparison of in vitro plantlets and ex vitro plants grown in the greenhouse on a one-to-one basis revealed that there was a significant individual correlation between the artemisinin content of paired in vitro plantlets and ex vitro plants grown in the greenhouse, with a very good correlation coefficient (R^2) value of 0.915 (**Figure 10a**). For plants transferred to the open field, the 10 in vitro-ex vitro pairs exhibited artemisinin contents for in vitro plantlets ranging from 0.25 to 0.69% and for ex vitro plants ranging from 0.31 to 0.84% (**Figure 10b**). A one-to-one comparison of the artemisinin content of plants grown in the open field with their corresponding plantlets also revealed a good R^2 value of 0.797 (**Figure 10b**).

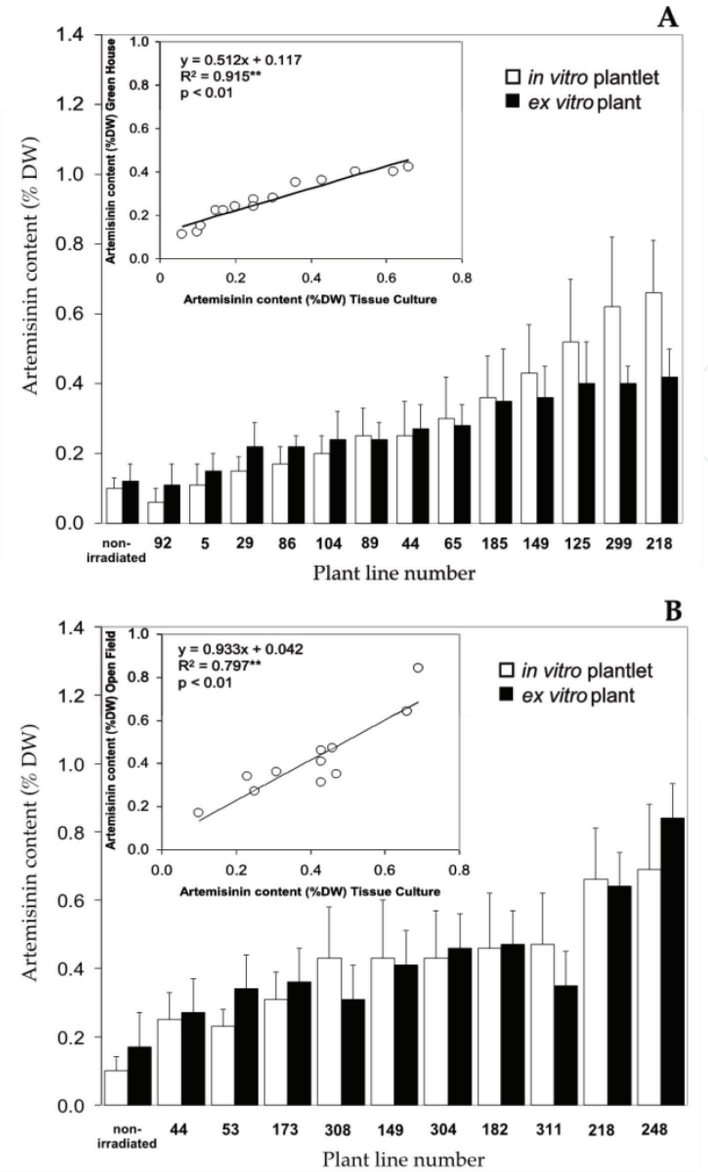

Figure 10.
One-to-one correlation of artemisinin content between in vitro gamma-irradiated plantlets and the ex vitro plants grown in a greenhouse showing a very good correlation coefficient value (R^2) of 0.915 (A, inset) and in an open field showing a relatively good R^2 value of 0.797 (B, inset).

3. Discussion

A. annua tissues appear to be sensitive to gamma rays, as a low-dose range (1–10 Gy) of radiation can generate viable plant variants. Within this range, variations in both plant growth and artemisinin content were observed in the resulting population of in vitro plantlets. Doses greater than or equal to 8 Gy, previously reported as the 50% lethal dose (LD_{50}) [20], clearly caused growth inhibition and leaf abnormalities in the plantlets, while the lower dose of 3 Gy resulted in growth promotion. The 5-Gy dose, on the other hand, has no significant effect on the plantlet morphology. Previous reports have indicated that low doses of gamma irradiation affect seed germination and seeding growth [24–27]. In red pepper (*Capsicum annuum*), three irradiated groups exposed to 2, 4, and 8 Gy exhibited enhanced seedling development [24]. In Arabidopsis, ultrastructural changes in cellular organelles have been observed after gamma irradiation, and seedlings treated with 0–5 Gy developed normally, while vertical growth in plants exposed to 50 Gy was significantly inhibited [27].

In terms of formation of secondary products, little information is available regarding the use of gamma irradiation for yield improvement. To our knowledge, the only related report characterized the effect of low-dose gamma irradiation (2–16 Gy) on the increased production of shikonin derivatives in callus cultures of *Lithospermum erythrorhizon* [28]. In agreement with these results, we also found a significant effect on artemisinin content caused by a similar low-dose range of gamma irradiation. However, in the present study, we observed these effects in mature plants, rather than in disorganized tissues. Our results show that treatment with either 8 Gy [19] or 5 Gy can create a population of plantlets with a high range of artemisinin contents. Due to the minimal associated morphological effects, the 5-Gy dose was chosen to produce *A. annua* variants that were presumably affected primarily at the biochemical and physiological levels.

The specific genes affected by the low-dose gamma radiation were observed to be at least on the gene of ADS of the biosynthetic pathway of artemisinin in *A. annua* plantlets. More than half of the variant population appeared to have a high correlation coefficient value (R = 0.922) between artemisinin content and ADS enzyme activity. The reason why ADS gene is particularly sensitive to the irradiation is still not clear. However, it might be that the low doses of 5–8 Gy of the gamma irradiation are just mild enough to affect this ADS gene. In principle, some lesions of ADS gene caused by the irradiation are likely to be repaired through the action of intracellular DNA repair process, while ADS gene of some other samples might remain unpaired or misrepaired, giving rise to permanent changes in the affected ADS gene. This would lead to a cellular response including a wide range of the enzymatic systems, as observed in this case with the variable ADS enzyme activities in *A. annua*. In the literature, there has been a report supporting our results. That is the case of low-dose irradiated callus cultures of *Lithospermum erythrorhizon* in which the enzyme activity of p-hydroxybenzoic acid geranyltransferase involving in the shikonin biosynthesis is boosted after the gamma irradiation [28]. Thus, it was suggested that the creation of plant variation through gamma irradiation has significant effect on ADS gene which is likely to be related to the enhancement of the artemisinin content in *A. annua*.

For the ex vitro acclimatization of the plants, we have previously characterized the conditions and supporting material important for photoautotrophic growth of *Eucalyptus camaldulensis* plantlets, both in vitro and ex vitro [23]. Adoption of this protocol resulted in a survival rate of 67% for *A. annua* plants after the in vitro-ex vitro transfer and a survival rate of 38% after 6 months of ex vitro growth to obtain mature plants. Clearly, the stresses generated in weakened irradiated plants during the process of acclimation lead to significant mortality. However, whether the mortality is more prevalent among in vitro individuals with low or high artemisinin content remains unclear.

For the 23 surviving mature plants, we observed an individual correlation in artemisinin content between the in vitro plantlets and the ex vitro mature plants. This one-to-one correlation was strongly positive for plants grown in the greenhouse, with $R^2 = 0.915$, and relatively positive for field-grown plants, with $R^2 = 0.797$. These results suggest that the capability for artemisinin biosynthesis in each in vitro plantlet is maintained throughout the in vitro-ex vitro transfer and the subsequent development into a mature plant. With respect to the greenhouse plants, although the correlation coefficient value was quite high, the high-yield plants did exhibit a reduction in artemisinin content. This observation is likely due to the high biomass weight per leaf for high-content leaves, which clearly appear thicker than low-content leaves found in greenhouse conditions.

The differences in biomass associated with artemisinin content are not so obvious among the established in vitro plantlets, resulting in a decrease in the

degree of the one-to-one correlation observed strictly for high-yield plants. For the field-grown plants, the relatively positive value of the correlation coefficient (R^2 = 0.797) may be due to two outliers present among the 10 samples (nos. 308 and 311) that deviate from the rest of the population. A pairwise comparison of the remainder of the samples would result in a higher R^2 value, which is reflective of a good correlation in artemisinin content between the in vitro plantlets and ex vitro field-grown plants. In addition, the lower correlation could also be attributed to the less controlled conditions of the open field compared with those of the greenhouse.

To be certain that changes in artemisinin biosynthesis in the ex vitro plants are genetically stable, it is necessary to test the next generation. However, many of the established mature plants could not produce seeds. Therefore, stability tests assessing the next generation through seed germination are not possible. Alternatively, this analysis can be performed through a second round of in vitro-ex vitro transfer. No attempt was made to use this method in the present study due to the high mortality associated with this long process that would have resulted in an insufficient number of pairs of plants for a one-to-one analysis.

4. Conclusion

Based on these results, we conclude that the technique of gamma irradiation can produce viable variants of *A. annua* that are capable of maintaining changes in gene expression associated with the artemisinin biosynthetic pathway (such as ADS) throughout the in vitro-ex vitro transfer process and, at minimum, through the first generation of mature plant development. Relatively low doses of gamma irradiation (ca. 3–8 Gy) can be effective for yield enhancement of artemisinin in *A. annua*. A mechanistic understanding of the increased biosynthesis of artemisinin in response to gamma irradiation is important for the development of a production-scale operation.

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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