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INTRODUCTION

Artemisinin (Figure 1) is an anti-malarial sesquiterpene lactone isolated from *Artemisia annua* L. (Asteraceae). The plant is a wild growing species with relatively low artemisinin content, ranging from 0.01–0.8% of the plant dry weight, depending on the geographical origin, seasonal and somatic variations[1]. Presently, the only commercial source of artemisinin is by extraction from field-grown leaves and flowering tops of the plant. Many attempts to obtain artemisinin in a cheaper way through chemical synthesis[2] and in *vitro* culture[3-6] have been made, but have yet to prove commercially feasible. Ionizing radiation has been recognized as a powerful technique for plant improvement.[7-8] 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.[9-10]

We have recently reported a method for establishing *in vitro* plantlet variants of *Artemisia annua* using low-dose gamma irradiation (1-10 Gy), together with plant tissue culture techniques.[11] Plantlets that survived treatment with 8 Gy radiation, the 50% lethal dose (LD_{50}), exhibited a wide variation in content of artemisinin.

In this study, the effect of two cycles of gamma-irradiation on the accumulation of artemisinin in the irradiated plantlets of *A. annua* was carried out in order to obtain information for the yield improvement of this anti-malarial sesquiterpene lactone in this plant.

MATERIALS AND METHODS

Plant material and culture media

Artemisia annua L. seeds, originating from Hanoi, Vietnam, were surface-sterilized and germinated on hormone-free MS medium supplemented with 3% sucrose and solidified with 0.8% agar. *In vitro* plantlets were established using previously described methods.[11] The resulting plantlets were subcultured five times prior to treatment with gamma irradiation.

Gamma ray treatment

Actively growing shoot tips (length, 5 ± 1 mm) were excised from *in vitro* plantlets and stripped of their leaves. The resulting shoot tips (130 tips) were then inserted vertically 2 ± 1 mm in depth into MS medium containing 3% sucrose and 0.25% Phytagel® from Sigma. Introduction of variation into *A. annua* plantlets using gamma irradiation was performed as previously reported.[11] In the present study, the shoot tips were treated with 5 Gy using gamma rays (Cobalt-60; dose rate, 6.85 Gy/min) at the Gamma Irradiation Service and Nuclear Technology Research Center, Kasetsart University (Bangkok, Thailand) and were then transferred to hormone-free MS medium for plantlet development. From the resulting subcultures, a selected shoot with two buds was excised from each plantlet. The upper bud was regenerated to from shoots, and the lower bud was regenerated to form roots. Leaves from healthy plantlets surviving after four subculture passages (90 plantlets) were then harvested for determination of artemisinin content, and shoots were used for a second round of plant regeneration.

Quantitative analysis of artemisinin content

Fresh leaves obtained from various *in vitro* irradiated plantlets (biomass sufficient for one sample per *in vitro* plantlet) were dried at 50°C in a hot-air oven. Each dried sample was ground into a powder, and artemisinin was extracted by refluxing in hexane (30 mg powder/5 mL hexane by the Syncore® Analyst, Buchi, Switzerland) as described previously.[11] The artemisinin content in each hexane extract was analyzed in triplicate using a densitometric thin layer chromatography (TLC) method. The TLC method was based on ammonia vapor-mediated structural conversion of artemisinin on a silica gel layer into a product that can be detected by ultraviolet (UV)-based densitometric TLC.[12] TLC plates were scanned under a wavelength of 310 nm using a Camag scanner II in combination with CAMAG, CATS V4.02 software (Muttenz, Switzerland). The area under the artemisinin peak for each sample was then converted

to artemisinin content based on a calibration curve that exhibited linearity from 0.06–12 μg/mL artemisinin. The resulting artemisinin quantities were used to calculate the content as a percentage of dry weight.

Statistical analysis

Values shown in graphs represent the mean ± standard deviation of triplicate measurements of a single extract for each *in vitro* plantlets. Correlation coefficients between the artemisinin content of the *in vitro* plantlets were calculated using SPSS software (SPSS Inc., Chicago, IL, USA).

RESULTS

Variation of artemisinin content in the irradiated plantlets of A. annua

With the 5 Gy treatment of gamma-irradiation, the surviving plantlets after four subsequent subcultures were analyzed for their ability to accumulate artemisinin. The experiments were carried out comparatively among three groups of plantlet population: the non-irradiated plantlets (control), the first generation and the second generation of the irradiated plantlets. The results showed that the control plantlets showed a narrow range of low artemisinin content (0.25 ± 0.04% dry weight) (**Fig. 1A**), whereas the first-generation irradiated plantlet population showed a significant higher value of the average artemisinin content (0.38 ± 0.13% dry weight) with a wide variation of artemisinin content from 0.12% to 0.59% (**Fig. 1B**). On the other hand, the second-generation population of the irradiated plantlets appeared to contain significant lower artemisinin than the control (0.1 ± 0.05% dry weight) (**Fig. 1C**). In terms of content distribution, the control group had 75% of the plantlets containing 0.2-0.3% artemisinin range (**Fig. 1A**), the first-generation group had 75% of the plantlets containing 0.3-0.6% (**Fig. 1B**), and the second-generation group had more than 90% containing less than 0.3% dry weight of artemisinin (**Fig. 1C**). These results were clearly shown as direct comparison in the same graph in **Fig. 2.**

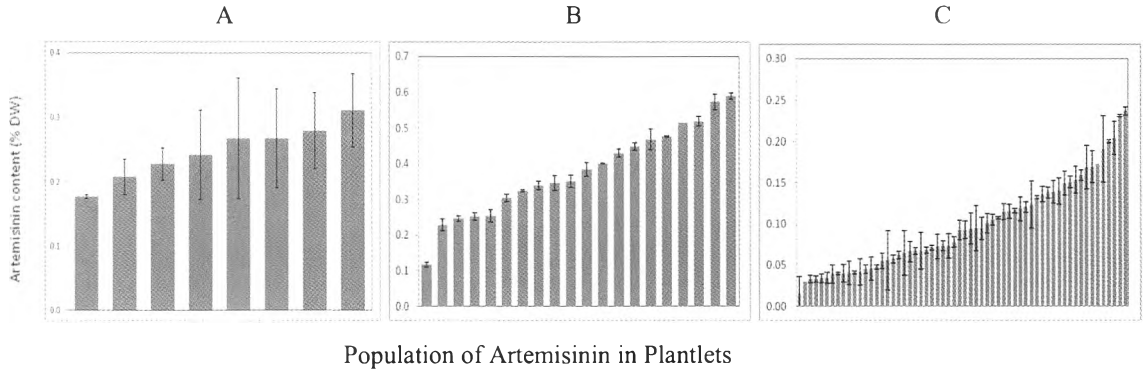

Figure 1 Variation of artemisinin content in various plantlets of *A. annua* that their shoot tips had been exposed to a dose of 5 Gy of gamma rays. A = non-irradiated control plantlets, B = first-generation irradiated plantlets and C = second-generation irradiated plantlets.Insets show distribution of the plantlet populations with respect to artemisinin content in the three sample groups.

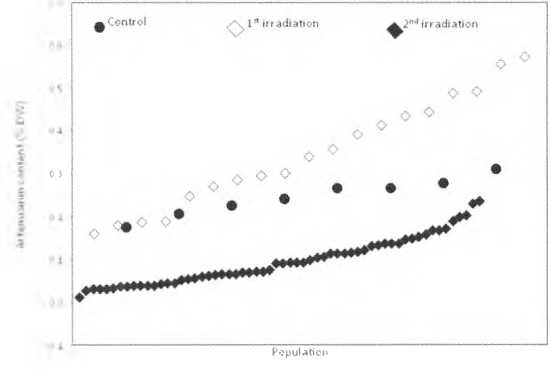

Figure 2 Direct comparison of artemisinin content distributed in the population of *A. annua* plantlets obtained from of the non-irradiated (•), first-generation irradiated (◊), and second-generation irradiated (♦) groups.

Effect of gamma irradiation on the plantlets germinated from the seeds of the first-generation irradiated plants

When the first-generation plantlets were grown in the field to obtain mature *A. annua* plants, it was found that the mature plants were still fertile and gave seeds. Interestingly, the obtained seeds (from the irradiated plant no.104) could still be germinated to give plantlet population with artemisinin content (0.04 ± 0.02 %dry weight). Repeat gamma-irradiation on one of these plants, however, gave rise to a plantlet population that contained very low artemisinin level (**Fig. 3**).

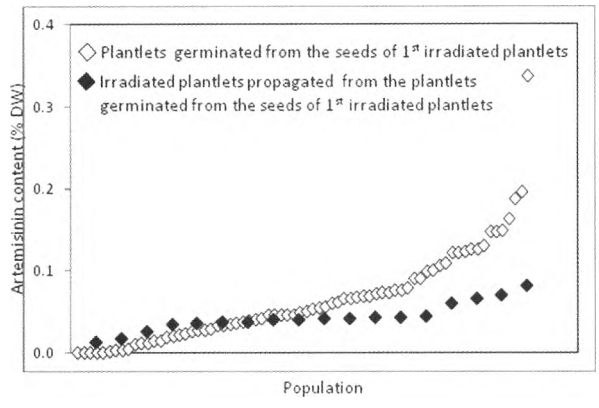

Figure 3 Effect of gamma irradiation on the plantlets germinated from the seeds of the first-generation irradiated plant.

DISCUSSION

We have shown here the effect of gamma irradiation on the in vitro plantlets of *A. annua* with respect to the accumulation of artemisinin in the leaves of two generations of the irradiation. It was concluded that the irradiation gave the positive effect on the first generation and negative effect on the second generation. Our experiments with the plantlets germinated from the seeds of the first-generation irradiated plants also support this trend. These results suggested that appropriate single dose of the gamma rays is important in the improvement of secondary metabolic expression in this plant, whereas repeat exposure of the plant with the radiation would decrease the expression. The irradiation effects on the cellular or subcellular functions are still not clear, although it is has been reported that there was a certain degree of correlation in the irradiated plants between the artemisinin content and the amorpha-4,11-diene synthase (ADS), the first enzyme of the artemisinin pathway.[11] Whether this correlation also be the case for the second generation of the irradiated plantlets remains to be studied.

CONCLUSION

1. Shoot tip irradiation using 5 Gy-dose of gamma rays causes the increase of average artemisimin content in *A. annua* leaves from 0.25% to 0.38% dry weight.
2. Repeat irradiation of the first-generation plantlets causes a dramatic decrease of the artemisinin content in the obtained second-generation irradiated plantlets (from 0.38% to 0.1% dry weight).
3. By starting from the seeds of the first irradiated plantlets, the irradiation also causes the considerable decrease of the artemisinin level in the leaves (from 0.06% to 0.04% dry weight).

ACKNOWLEDGMENTS

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