CHAPTER V

DISCUSSION

1. The Effect of Gamma Irradiation on the Development of *A. annua* Plantlets

When one thousand *A. annua* plantlets were exposed with gamma-ray from ^{60}Co in the range of 0-10 Gy, it was found that the doses below 5 Gy gave the plantlets with more leaves, stems and roots whereas the doses above 5 Gy gave small plantlets. As shown in Figure 17, the plantlets from the 8 Gy dose showed some apparent leaf abnormalities whereas the treatment with 5 Gy gave almost 70 % survival rate and the resulting plantlets were mostly in good morphological appearance. In addition, we showed previously that the plantlet population obtained from this 5 Gy dose contained artemisinin in a wide range from 0.02 to 0.67 % dry weight, with less than 10% of the plantlets containing artemisinin more than 0.5% dry weight (Koobkokkruad *et al.*, 2007).

In principle, gamma ray is electromagnetic (EM) radiation with high radiation energy. It has been showed that the gamma irradiation produced the reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2), superoxide anion ($O_2^{\cdot-}$), hydroxyl radicals (·OH) and singlet oxygen by water radiolysis (De Vita *et al.*, 1993, Dubner *et al.*, 1995, Kovács and Keresztes, 2002, Luckey, 1980, Miller, 1987 and Quintiliani, 1986). These can lead to damage covalent bonding, hydrogen bonding and/or other molecular bondings of biomolecules in the cell and thus causing chromosome

damage, gene damage (mutation), and eventually cell death (Alberts *et al.*, 2002). In plant metabolism, these radicals can damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry, and physiology of plants depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism, e.g., dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system, and accumulation of phenolic compounds (Kim et al., 2004, Kovács and Keresztes, 2002 and Wi et al., 2005). Generally, the damage of protein and RNA molecules can be quickly replaced using information encoded in the DNA but the DNA molecules themselves are irreplaceable. Therefore, maintaining the integrity of the information of DNA is a cellular imperative and supported by elaborate set of DNA system. Although most such lesions are normally repairable through the action of intracellular DNA repair process, those that remain unpaired or are misrepaired may give rise to permanent changes in the affected gene (mutation) or in the chromosomes on which the genes are carried. The cellular response of this change includes a wide range of the enzymatic systems that catalyze some of the most interesting chemical transformations in DNA metabolism and the other cellular metabolite both primary and secondary metabolite (Alberts *et al.*, 2002). With respect to morphological and biological responses, it has been reported very recently that seedlings exposed to relatively low doses of gamma rays (1–5 Gy) developed normally, while the growth of plants irradiated with a high dose gamma ray (50 Gy) was significantly inhibited (Wi *et al.*, 2007). For our study, we also observed the morphological changes of the plant especially the leaves which showed different characters among the population of the irradiated plantlets.

Recently, physiological symptoms in a large range of plants exposed to gamma rays have been described by many researchers (Kim *et al.*, 2004; Kovács and Keresztes, 2002 and Wi *et al.*, 2005). The studies observed the effect of the low- or high-doses of the variants on the inhibited or enhanced response for germination, seedling growth, and other biological responses (Kim *et al.*, 2004; Wi *et al.*, 2005). Kim and coworkers investigated alterations in the photosynthesis and antioxidant capacity of red pepper (*Capsicum annuum* L.) seedlings produced from gamma-irradiated seeds. For two cultivars (Yeomyung and Joheung), three irradiated groups (2, 4, and 8 Gy, but not 16 Gy) showed enhanced development, although the maximum photochemical efficiency of Photosystem II (PSII) did not differ significantly among any of the four groups. (Kim *et al.*, 2004). The ultrastructural changes of the cell organelles of *Arabidopsis* stems in response to gamma irradiation have been reported (Wi *et al.*, 2005). Seedlings treated with 0 to 5 Gy developed normally, while height growth in plants exposed to 50 Gy was significantly inhibited. In our studies, it was found that the irradiation doses of 8-10 Gy significantly inhibited the growth of the variants.

With respect to secondary metabolism, Benoit and co workers have reported the effect of gamma irradiation on mushroom (*Agaricus bisporus*) (Benoit *et al.*, 2000). The ionizing treatments at 0.5 kGy, 1.5 kGy and 2.5 kGy showed on significant increase of the phenylalanine ammonia-lyase activity and total phenols concentration. Very recently, the effects of low-dose gamma-irradiation on production of shikonin derivatives in callus culture of *Lithospermum erythrorhizon* has been reported (Chung *et al.*, 2006). They found that gamma irradiation significantly stimulated the shikonin biosynthesis of the cells and increased the total shikonin yields (intracellular+extracellular shikonin yields) by 400% at 16Gy and by

240% and 180% at 2 and 32 Gy, respectively. p-Hydroxylbenzoic acid geranyltransferase(PHB), one of the key enzymes for the shikonin biosynthesis of cells, was found to be stimulated by the gamma-radiation treatments. The activity of PHB geranyltransferase was increased at 2 and 16 Gy with a negligible change at 32 Gy. In contrast, the activity of PHB glucosyltransferase was slightly changed at all doses of gamma radiation compared with the control cells. Therefore, the increase in PHB geranyltransferase activity leads to the accumulation of secondary metabolites such as a shikonin, which may contribute to plant defense against the stresses induced by gamma irradiation.

Similarly, in our studies, we have shown that the 90 samples of the *in vitro* plantlets obtained from this dose contain artemisinin in a wide range from 0.02 to 0.67 % dry weight, with less than 10% of the plantlets containing artemisinin more than 0.5% dry weight (Koobkokkruad et al., 2007). The key enzyme in artemisinin biosynthesis in the irradiated variants, amorpha-4,11-diene synthase (ADS), the activity of ADS has also been shown to be related to the artemisinin content (Koobkokkruad *et al.*, 2002).

2. The Transfer of *In vitro* Plantlets to *Ex vitro* Conditions for Plant Development

In the photomixotophic step, the irradiated plantlets were maintained on MS medium containing 3 % sugar and 0.8 % agar and kept under 40 $\mu mol.m^{-2}.s^{-1}$ of fluorescence light. During the subculture, a selected shoot with two buds was excised from each plantlet. The bud at the top was regenerated to from shoots and the below bud was regenerated to form roots. The formation of shoots and roots after the subculture is an important step for the success of micropropagation which can be

evaluated from the value of the percent of plantlets that are successfully transferred from tissue culture vessels (*in vitro*) to *ex vitro* conditions of green house or field conditions. Thus, the plantlet with complete shoot and root was selected to transfer to acclimation conditions.

During the process of acclimation, the main causes that make plantlets loss *ex vitro* would be through foliar water loss (Ziv 1979: Shackel *et al.*, 1990) and restriction of water uptake by roots (Preece and Sutter, 1991). The situation is intensified by the culture-induced phenotype as well as the physiology, morphology and anatomy of leaves and roots developed *in vitro* (Grout and Aston, 1978; Donnelly *et al.*, 1986). These characteristics are different from those of greenhouse- or field-grown plants. Another cause that makes the loss during acclimation is insufficient photosynthetic capacity to provide a positive carbon balance after transplanting during the transition from photomixotrophic to photoautotrophic metabolism (Gruot, 1978; Gruot and Aston, 1978; Preece and Sutter, 1991). These factors were, therefore, considered during our *in vitro-ex vitro* transfer of the irradiated plantlets of *A. annua*.

After 6-week culture under *in vitro*, some selected irradiated plantlets were transferred into the photoautotrophic condition in 16 oz glass bottle contained 50 ml vermiculite that absorbed 50 ml MS medium without sucrose. One membrane filter (Milli-Seal, Millipore, Japan; pore size: 0.5 μm) was attached over a hole (area: 0.8 cm^2) on the plastic cap for increase the number of air exchange of the culture vessel. During the acclimation step, all the cultures were grown at 25±1 °C under 40 $\mu mol.m^{-2}.s^{-1}$ photosynthetic photon flux density provided by cool-white fluorescence lamps. It has been reported by Kirdmanee and co-worker on the effect of CO_2 enrichment and supporting material *in vitro* on photoautotrophic growth of *Eucalytus* plantlets *in vitro* and *ex vitro* (Kirdmanee *et al.*, 1995). In the report, the growth and net

photosynthetic rate of plantlets *ex vitro* were evaluated and found that CO_2 enrichment significantly increased the growth. For the supporting material, vermiculite was found to be best for supporting growth. In addition, the extensive root system produced *in vitro* was found to be necessary for growth and survival of plantlets *ex vitro*. Therefore, these optimized conditions were developed for this *in vitro-ex vitro* transfer of the irradiated *A. annua* plants.

3 Analysis of Artemisinin Content in the *In vitro* Plantlets and *Ex vitro* Plants of *A. annua*

When some selected plantlets with different artemisinin content were transferred from their *in vitro* cultures to the *ex vitro* conditions of both open-field and greenhouse, it appeared that the mature plants contained artemisinin in similar patterns to the original plantlets, and clearly with individual correlation of their biosynthetic potentials. These results suggested that the variation of artemisinin production in the irradiated plantlets of *A. annua* was essentially maintained after *ex vitro* transfer and, thus, stable high artemisinin-yielding plants of *A. annua* can be obtained by gamma irradiation.

The artemisinin content in *in vitro* culture of *A. annua* has been reported previously. The first trail of using a tissue culture technique for artemisinin production was from the report of He *et al.*, (1985). That report showed artemisinin content at 0.063 % DW for shoot cultures cultivated in MS medium containing 2 mg/l BA (Martinez and and Staba, 1988), Subsequently, Simon *et al.*,(1990) have reported the contain in range of 0.03-0.05 % DW. In 1992, artemisinin content has been reported to contain 0.012 % DW in plantlets cultures with MS medium containing 1

ppm NAA and 0.1 ppm kinetin (Elhag *et al.*, 1992), 0.08-0.16 % DW for shoot culture established on MS medium containing 0.2 mg/l BA and 0.05 mg/l NAA (Woerdenbag *et al.*, 1993). Ferreira *et al.*, (1993) have reported that the artemisinin content in range of 0.014-0.290 % DW was detected *in vitro* culture plantlets. In this study, while mean artemisinin content of the *in vitro* irradiated plantlets was found to be 0.366 % DW (with a wide range from 0.09 to 0.69 % DW) (Figure 18). The value of 0.69 % is higher than the results reported before in literatures.

Our data analysis of the artemisinin content between the *in vitro* plantlets and the *ex vitro* plants in the green house showed that there were relatively high degree of correlation. As shown in Figure 18, the *in vitro* plantlets contained artemisinin in range of 0.06 to 0.66 % DW (mean = 0.30) and the *ex vitro* plants in the green house contained in the range of 0.12 to 0.42 % DW (mean = 0.27) (Figure 24). Our results are, however, in contrast with the results of higher artemisinin content for plants grown in *in vitro* (Pras *et al.*, 1991) and in green house (Elhag *et al.*, 1992) compared with open field grown plants. The work of Pras *et al.*, (1991) showed no correlation of artemisinin content between laboratory- and field-growth conditions. However, our results appear to be similar to Ferreira *et al.*, (1994) that have reported the relationship of artemisinin content between tissue culture, green house and field grown plant of *A. annua*, although they no report the correlation of artemisinin content between *in vitro* plantlets and field growth conditions. Therefore, with those results and our report from this study, it is suggested that the artemisinin content in each irradiated *A. annua* plantlets is maintained during its transferred from *in vitro* (photomixotophic) plantlets to *ex vitro* (photoautotrophic) and in open filed.

4. Cloning and Expression of *Amorpha-4,11-Diene Synthase* Gene

This is the first study on the over expression of *ads* gene in *A. annua*. The results showed that amopha-4,11-diene synthase gene from *A. annua* was composed of 1,638 nucleotides. The deduced amino acid sequence of open reading frame was 546 amino acids (Figure 30). The *ads* gene was inserted into pBI121 plant expression vector under a CaMV 35s promoter. *A. tumefaciens*-mediated transformation system was developed for *A. annua*. Using this system a cDNA encoding ADS was transferred into *A. annua* via *A. tumefaciens*. Subsequent analysis of PCR showed that the shoots of *A. annua* transferred with the 35s promoter were PCR positive. The ADS activity in the transgenic *A. annua* was 2-3 times higher than the untransgenic *A. annua*. This led to an observed increase of artemisinin content to 0.8-1.0 % DW which is also about 2-3 times higher than the control.

Previously, Bouwmeester *et al.*, (1999) have reported the presence of amorpha-4,11-diene in pentane extracts of *A. annua* leaves. Their findings have led to conclusion that amorpha-4,11-diene synthase is the enzyme catalyzing the first step of artemisinin biosynthesis. The enzyme converts farnesyl pyrophosphate into amorpha-4,11-diene. The primers designed for cDNA synthesis of our study were downloaded from previous reports (Wallaart *et al.*, 2001; Chang *et al.*, 2000; Merck *et al.*, 2000). The aliments of deduced amino acid sequence of ADS were performed. The ADS amino acid sequences were download from GenBank included AY006482 (Wallaart et al. 2001), AJ251751 (Chang *et al.*, 2000), DQ241826 (unpublished), EF197888 (unpublished) and AF138959 (Merck *et al.*, 2000). It was found that the aliment scare between our ADS amino acid sequence were 98.53, 98.71, 97.80, 98.53 and 98.71, respectively (Figure 31). These suggested that the amino acid sequence confirmed

this sequence was encoded from *ads* gene. Some amino acid positions of the aliment were different but have no enough results in the enzyme activity that may be relate to artemisinin production.

The ADS enzyme has been partial purified and shows the same typical characteristics of sesquiterpene synthase (Bouwmeester *et al.,* 1999). The enzyme product has been shown to be present in low content in the plant *A. annua*. Because the low abundance of the amorpha-4,11-diene, it has been suggested that the cyclization of FDP is a rate-limiting step in artemisinin biosynthesis (Bouwmeester *et al.,* 1999). The cDNA encoding of amorpha-4,11-diene synthase has been isolated from *A. annua* genome (Mercke *et al.,*2000). This clone contains a 1641-bp open reading frame coding for 546 amino acids (63.9 kDa). When expressed in *Escherichai coli*, the recombinant enzyme has been shown to catalyze the formation both olefinic (97.5 %) and oxygenated (2.5 %) sesquiterpenes from farnesyl pyrophasphate. GC-MS analysis has identified as 91.2 % amorpha-4,11-diene. Moreover, the enzyme amorpha-4,11-diene synthase has been cloned by PCR amplification of genomic DNA with a pair of primers, which designed from the conserved region of sesquiterpene synthase of several plants (Chang *et al.,* 2000). The PCR technique has produced 2106 bp of a full length the cDNA sequence including 1641 bp of open reading frame for 546 amino acid.

The soluble fraction of *E. coli* harboring of the gene has been shown to catalyze the cyclization of fanesyl pyrophosphate to produced a sesquiterpene, which is identified through GC-MS analysis as amorpha-4,11-diene (Chang *et al.,* 2000). Wallaart *et al.,* (2001) have reported the isolation of a cDNA clone encoding amorpha-4,11-diene synthase. When the *ads* gene was expressed in *E. coli*, the recombinant enzyme catalyses the formation of amorpha-4,11-diene from farnesyl

pyrophasphate. In addition, the tranformation of the gene into tobacco (*Nicotiana tabacum* L.) has resulted in the expression of an active enzyme and the accumulation of amorpha-4,11-diene ranging from 0.2 to 1.7 ng per gram fresh weight. The engineering of the mevalonate pathway in *E. coli* have been reported in 2003 (Martin *et al.*, 2003). This reseach group engineered the expression of a synthetic *ads* gene and the mevalonate isoprenoid pathway from *Saccharomyces cerevisiae* in *E. coli.* The strain developed in this study can serve a platform hosts for the production of any terpenoid compound for which a terpene synthase gene is available. In addition, Ro *et al.*, (2006) have reported the engineering of *S. cerevisiae* to produce high titres (up to 100 mg/l) of artemisinic acid using an engineered mevalonate pathway, amorphadiene synthase, and a novel cytochrome P450 monooxygenase (*CYP71AV1*) from *A. annua* that performs a three-step oxidation of amorpha-4,11-diene to artemisinic acid. Although, the engineered yeast is already capable of producing artemisinic acid at a significantly higher specific productivity than *A. annua*, yield optimization and industrial scale-up will be required to raise artemisinic acid production to a level high enough to reduce artemisinin combination therapies to significantly below their current prices.

The overexpression of *ads* gene in *E. coli*, *S. cerevisiae* and tobacco have no the other enzymes in artemisinin biosynthesis that flow the amorpha-4,11-diene intermediate to artemisinin. Therefore, the results of their studies have no artemisinin present in these organisms.

On the other hand, the transformation of *A. annua* plant by using *Agrobacterial rhizogenas* have been reported in transformed hairy roots, but not in transgenic plants (Weathers *et al.*, 1994; Jaziri *et al.*, 1995; Paniego and Giuletti, 1996; Qin *et al.*, 1994). Vergauwe *et al.*, (1996) has established an *A. tumefaciens-*

mediated transformation system, and showed that artemisinin content in leaves of regenerated plantlets was 0.17 % DW, a little higher than that present in leaves of *in vitro* plantlets (0.11 % DW). These works showed the parallel with our results in case of regenerated *A. annua* plantlets, but have no transgenic plantlets.

In order to develop high inducing frequency shoot culture for transformation, different combination hormone concentration were used MS medium with only TDZ that showed the highest percentage of regenerated shoots. In the series of experiments, we compared the effect of only TDZ in MS medium at several concentrations. The shoots were regenerated after subculture for 2 weeks on MS medium with TDZ 0.1 mg/l and then transferred onto hormone-free MS medium (Wanwimon Luealon, 2006). Previously, the highest shoot regeneration was obtained as 0.5 μM TDZ with MS medium for micropropagation of *Menthax piperita* L. (78.3 %) (Faure *et al.*, 1998). Prathanturarug *et al.*, (2003) have reported the number of shoots per explant of *Curcuma longa* L. cultured on MS medium with 18.17 μM TDZ (18.22±0.62). Moreover, the effects of NA/BA in this condition were studied. The regenerated plantlets from MS medium were supplemented with 0.05 mg/l NA plus 0.5 mg/l BA and 1 mg/l NAA plus 1 mg/l BA. In the previous report, Vergauwe *et al.*, (1996) has performed the combination hormones of 0.05 mg/l NAA plus 0.5 mg/l BA. In contrast, stem segments were cultured in our laboratory and better result were reported when compared to leaf disks. Furthermore, Jun-li et al (2004) has studied high efficiency of genetic transformation and regeneration of *A. annua* via *A.tumefaciens*-mediated system. It was found that the highest shoot induction frequency of *A. annua* strain NJ was 0.05 mg/l NAA and 0.5 mg/l BA; *A. annua* strain 001 was 0.05 mg/l NAA 1.0 mg/l BA.

The genetic engineering of the *ads* gene overexpression in this study used the binary plant expression vector pBI121/*ads* insert in *A. annua* chromosome via *A. tumefaciens*-mediated transferred. The high level of amorpha-4,11-diene synthase expression under a CaMV 35s promoter let to the high level conversion of farnesyl diphasphate to amorpha-4,11-diene and then flow to complete artemisinin compound. Our study showing the high level of ADS specific activity (cpm, mg protein^{-1},min^{-1}) confirmed that the effect of plant expression vector showed 2-3 folds from untransgenic *A. annua.* The results of TLC-densitometric analysis showed that artemisinin production in the transgenic plants had the yield of 1.0 % dw.

The improvement of artemisinin production by mean of cell or tissue culture has not been achieved as yet. It would, therefore, be interesting to produce transgenic plants of *A. annua* which ensure a constant high artemisinin production by overexpression of the enzyme(s) of the terpene biosynthetic pathway or inhibiting an enzyme(s) of another pathway competing for its precursor.

In the isoprenoid biosyntheic pathway the carbon flow starting from the C15-FDP may flow to different pathways. One leads to sesquiterpenes catalyzed by sesquiterpene synthase, the others to sterols and saponins catalyzed by squalene synthase. It has been reported that the squalene synthase activity was suppressed, while the biosynthesis of sesquiterpenoid phytoalexin is induced, when fungal elicitors were added to tobacco suspension culture (Vogeli and Chappell, 1990). Therefore, a simplistic model had been assumed that the FDP was equally available to both sqalene synthase and sesquiterpene synthase, and that sesquiterpeniods and sterol biosynthesis are not localized to different subcellular compartment.

There have been reports that give emphasis on the enzyme FDP to be important for artemisinin synthesis. Chen *et al.,* (2000) have transferred a cDNA

encoding FDP and placed under a CaMV 35s promoter into *A. annua*. Analysis of artemisinin showed that artemisinin content increase about 3-4 times from the control shoots in range of 1.0 % DW. These studies suggested that although manipulation of FPS increased artemisinin, the yields were still not adequate to significantly increase artemisinin production in plants.

In addition, the effect of plant growth regulators on artemisinin formation in *A. annua* has been studies. Artemisinin content has been shown to increase yield significantly when gibberellic acid (GA_3) was added in plant growth conditions (Farooqi *et al.*, 1996; Siyapatantakirutimana *et al.*,1996). Moreover, biomass of the plant has been observed to increase with GA_3 treatment (Siyapatantakirutimana *et al.*, 1996). In hairy root culture of *A. annua*, the effect of GA_3 has been shown to have significant increase in biomass and in artemisinin content (Smith *et al.*, 1997).

Therefore, for the future work, the important of artemisinin yield should be emphasized on the overexpression enzymes regulation in the artemisinin biosynthesis. This should combine our work with other steps of key enzymes in the artemisinin partway. In this investigation, *ads* gene under overexpression 35s promoter was transferred into *A. annua* by using an *A. tumefaciens*-mediated transformation and showed that the overexpression of ADS recombinant gene could increase accumulation of artemisinin in transgenic *A. annua*.