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Original Paper

Overproduction of artemisinin in tetraploid *Artemisia annua* L.

Waleerat Banyai[1,2], Ratchada Sangthong[2], Netiya Karaket[1], Phithak Inthima[1], Masahiro Mii[2], Kanyaratt Supaibulwatana[1,*]

[1] Department of Biotechnology, Faculty of Science, Mahidol University, Bangkok 10400, Thailand; [2] Laboratory of Plant Cell Technology, Graduate School of Horticulture, Chiba University, Matsudo, Chiba 271-8510, Japan
* E-mail: scksp@mahidol.ac.th Tel: +66-0-2201-5303 Fax: +66-0-2354-7160

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Abstract Tetraploid plants of *Artemisia annua* L. exhibiting high-artemisinin-yield were successfully induced by treating excised leaves of *in vitro* plant with 0.1% colchicine. The chromosome number of original diploid plant was confirmed to be $2n=2x=18$ whereas that of the tetraploid plant was $2n=4x=36$. Morphological and anatomical characteristics of tetraploid plants were obviously different from the diploid counterpart. The tetraploid plants had larger sizes of root system, stomata and glandular secretory trichomes than diploid plant, whereas leaf size in tetraploid was smaller but thicker than diploid. The highest amount of artemisinin content was produced at flower blooming stage in both diploid (2.4% dry weight) and tetraploid (3.8% dry weight) lines. Based on the maximum yield of artemisinin, the optimum harvest time for the diploid lines was flower initiation stage but it was at full blooming stage for tetraploid lines. The highest artemisinin yield in tetraploid plants up to 3.0 mg plant^{-1} was detected at this stage which was 1.5 times greater than diploid plants. This is a first report that clearly showed the potential of chromosome doubling strategy to produce high-yield line of *A. annua* plant for artemisinin production.

Key words: *Artemisia annua* L., artemisinin, chromosome, colchicines, tetraploid.

Artemisinin has been identified as a terpenoid, an endoperoxide sesquiterpene lactone that is effective against drug-resistant malaria. Artemisinin-based combination therapies (ACTs) were recommended by World Health Organization (WHO) to be the best choice for acute malaria, especially for all countries previously experiencing the resistance of *Plasmodium falciparum* parasites (Mutabingwa 2005). Approximately 100 million ACT treatments have been required annually in the world (Covello 2008). Recently, artemisinin and its derivatives have also been found to have the potential for the treatment of other infectious diseases such as schistosomiasis, hepatitis-B and leishmaniasis (Romero et al. 2005; Sen et al. 2007; Xiao 2005) and the effectiveness against cancer cell lines (Efferth 2006; Singh and Lai 2001). However, artemisinin is produced in leaves of *Artemisia annua* plant in small amounts of 0.01–0.8% of dry weight (DW) and the content varies depending on genotype and cultivation conditions (Abdin et al. 2003). The imbalance of demand and supply has led to the expensive cost of artemisinin-based drug that is the major obstacle for commercialization (White 2008). Clone improvement through genetic manipulation is one of the attractive strategies to potentially supply the necessary demand of artemisinin.

The enhancement of the secondary metabolite productions via induced polyploidy was previously demonstrated (Dhawan and Lavania 1996; Zhang et al. 2010). Chromosome doubling can be artificially induced by using the toxic natural product, colchicine, which acts to inhibit chromosome segregation during cell division. This type of genetic manipulation is frequently used in commercial breeding of plants (Otto and Whitton 2000). The productions of tropane alkaloids in *Atropa belladonna* and quinine in *Cinchona succirubra* were 68% and 100% increased, respectively, in the tetraploid lines compared with the original diploid lines (Evan 2009). The alkaloid content in tetraploid seeds of *Datura innoxia* and *D. stramonium* was about 2-fold higher than their diploid seeds (Berkow 2001). The improvement of flavonoids and terpenoids contents (per gram of tissue) has previously been reported in tetraploids of *Chamomilla recutita*, Petunia 'Michell' and *Salvia miltiorrhiza* (Gao et al. 1996; Griesbach and Kamo 1996; Svehlikova and Repcak 2000).

In *A. annua*, tetraploid hairy root was induced by colchicine treatment in the hairy root clone YUT16 (Jesus-Gonzalez and Weathers 2003). The polyploid hairy root clone YUT16-8LT produced artemisinin up to six times ($\mu g\,g^{-1}$ DW) more than their diploid line.

Abbreviations: ACT, Artemisinin-based combination therapy; ART, artemisinin; BA, N^6-benzyladenine; DW, dry weight; FW, fresh weight; GST, glandular secretory trichome; NAA, α-naphthaleneacetic acid; PPF, photosynthetic photon flux density.
This article can be found at http://www.jspcmb.jp/

Unfortunately, however, the diploid still grew better than the tetraploid and the increased yields of tetraploid have not reached to the commercially useful quantities of artemisinin (Jesus-Gonzalez and Weathers 2003). The induction of the polyploid in *A. annua* whole plant, from 18 to 36 chromosomes by colchicine treatment was previously demonstrated by Wallaart et al. (1999), who showed that the artemisinin level in tetraploid plant was 38% greater than that of the diploid. However, the biomass was lower than that of the diploid because the tetraploid plant was proportionately smaller. Therefore, there is no significant difference of the overall yield of artemisinin per plant between diploid and tetraploid plants. Therefore, the production of tetraploid *A. annua* with normal or high growth rate compared with its diploid line is interesting to investigate.

The objectives of this research were to produce the tetraploid *A. annua* by colchicine treatment and to evaluate artemisinin production and plant biomass in tetraploid plant under different developmental stages.

Materials and methods

Plant materials

Plant material used throughout this study is *Artemisia annua* L. (Qing-hao) that is originally obtained from Vietnam. Seeds were surface-sterilized with 0.25% sodium hypochlorite solution (Clorox®) and germinated on 0.7% agar-solidified MS medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and incubated under 25 ± 2°C, 60 ± 5% relative humidity and 16 h photoperiod with photosynthetic photon flux density (PPF) of $60 \pm 5\,\mathrm{mmol\,m^{-2}\,s^{-1}}$. Germination was observed within 4–5 days, and shoot segments, each with 2–3 nodes, were excised from 30 day-old seedlings and sub-cultured for multiplication by transferring regularly onto the same medium every 30 days.

Induction of polyploidy in A. annua

Detached *in vitro* leaves from 2 week-old *in vitro* plantlets (code no. 500) were immersed in the 0, 0.01, 0.1 or 1 (% w/v) filtered-sterilized colchicine solution for 24 h at room temperature (25 ± 2°C). The treated leaves were briefly washed with liquid MS medium prior to culture on regeneration medium (solidified MS basal medium supplemented with 0.1 $\mathrm{mg\,L^{-1}}$ NAA and $1\,\mathrm{mg\,L^{-1}}$ BA, pH 6.0). The regenerated shoots were transferred onto plant growth regulator (PGR)-free MS medium for root induction and sub-cultured for multiplication by transferring regularly onto the same medium composition every 30 days. The tetraploid code no. 5GC selected among the lines originated from different tissues was transplanted to *ex vitro* culture to compare their growth ability and artemisinin production with its diploid original line.

Flow cytometric analysis

Flow cytometry was applied to quantify DNA content of the putative tetraploid clone according to the method of Mishiba and Mii (2000) using a Partec PA cytometer equipped with a mercury lamp (Partec, Germany). Ploidy level was determined by comparing the position of dominant peaks corresponding to nuclei at G0-G1 phase of the cell cycle, between tetraploid and diploid plant. To release nuclei, approximately 0.1 g fresh weight (FW) of the sample tissues was collected and chopped with a razor blade in 0.3 ml of solution A of plant high-resolution DNA kit type P (Partec, Germany) in a plastic Petri dish (Galbraith et al. 1983). After incubating the crude nuclei suspension for 5 min at room temperature, 1.5 ml of staining solution composed of 10 mM Tris, 50 mM sodium citrate, 2 mM $MgCl_2$, 1% (w/v) Polyvinylpyrrolidone (PVP), 0.1% (v/v) Triton X-100 and $2\,\mathrm{mg\,l^{-1}}$ 4′,6′-diamidino-2-phenylindole (DAPI), pH 7.5, was added to the crude suspension and filtered through a 30-μm nylon mesh. After 5 min of staining, the suspension of nuclei was subjected to Flow cytometric analysis for determining the relative nuclear DNA content on a semi-logarithmic scale histogram.

Chromosome observation

Root tip meristems were excised from *in vitro* cultures of both diploid and tetraploid plants. The meristems were pretreated with 2 mM 8-hydroxyquinoline aqueous solution at 20°C for 3 h. The samples were fixed in absolute ethanol and glacial acetic acid (3 : 1) for 2 h at room temperature and stored in the fixative at 4°C for 24–48 h. The treated materials were hydrolyzed in 1M HCl for 5–8 min at 60°C. They were then stained with 1% aceto-orcein solution for at least 24 h, and squashed on slides in 45% acetic acid-glycerol (9 : 1). The chromosomes were observed with an optical microscope at a magnification of 1,000× and the best metaphase views were photographed with digital camera (Nikon, Japan). At least ten samples were analyzed for each clone.

Characterization of guard cell, leaf thickness and glandular secretory trichome (GST)

Leaflets were excised from different plantlets, and the lower epidermis of each leaflet was peeled with a tape and fixed onto a slide glass. Number and morphological characteristics of the guard cell in the fixed epidermis were observed and photographed under TM-1000 microscope (Hitachi High-Technologies Co., Tokyo, Japan). Measurement and observation of leaf thickness and GSTs were also performed under the microscope.

Plant cultivation and transplantation

Two lines of *in vitro* plants of *Artemisia annua* L., diploid plant code no. 500 and tetraploid plant code no. 5GC, were maintained on 0.7% agar-solidified MS medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose and incubated under 25 ± 2°C, 60 ± 5% relative humidity (RH) and 16 h photoperiod with PPF at $60 \pm 5\,\mathrm{mmol\,m^{-2}\,s^{-1}}$. Shoot segments were excised from 30 day-old plantlets and sub-cultured for multiplication by transferring regularly onto the same medium every 30 days. The *in vitro* plantlets (~5-cm height) were acclimated by transferring to photoautotrophic conditions each line with 30 plants. Under aseptic photoautotrophic culture, vermiculite filled with liquid MS medium (PGR-free and sugar free) was used as the supporting material. Air ventilation and CO_2 exchange into cultured vessels were promoted through 45 μm-filter membranes (ϕ=1 cm) that were applied to each vessel cap. The cultures were

acclimated for 2 weeks prior to transplant to pots containing mixed soil (Klasman®, UK) and then incubated in plant growth chamber (model GS1700CH, Giant Star Ltd., Korea) under 25 ± 2°C, 60 ± 5% RH, 16 h photoperiod with 80 ± 5 μmol $m^{-2} s^{-1}$ PPF, to observe growth and morphological characters. A short photoperiod (8 h/d) was applied to plants after transplantation for 1 week in order to induce flowering. Leaves were collected for artemisinin analysis at the vegetative stage (28 days after treatment), the flower initiation stage (>50% of the plants showing signs of inflorescence initiation) and the flower blooming stage (>70% of the flowers already open).

Artemisinin evaluation by HPLC

Leaves were excised and immediately dipped into liquid nitrogen and subsequently stored at -80ºC till the use for the artemisinin analysis. Crude artemisinin was extracted according to the protocol modified from that of Van Nieuwerburgh et al. (2006). One gram of sample was dipped into 10 mL chloroform and immediately vortexed for 1 minute. The solvent was evaporated in a fume hood at room temperature until absolutely dried prior to use as a crude extract. The artemisinin content was evaluated according to the procedure of Vandenberghe et al. (1995) with some modifications. Briefly, the crude extract was dissolved with 1 mL of methanol and then 200 μL of crude extract solution was separated into a new reaction tube. Exactly 800 μL of 0.2% (w/v) sodium hydroxide solution was added to the tube and mixed with a vortex mixer before incubation in a water bath at 50°C for 30 minutes. After cooling, 200 μL of methanol and 800 μL of 0.05 M acetic acid were added. The mixture was filtrated through a 0.45 μm Sartorious® membrane and then 10 μL of filtrate was subjected to HPLC (Water 717, USA) using a Luna 5 μm C18 100A column (150 mm×4.6 mm^2, Phenomenex, USA) with 1 mL min^{-1} flow rate. A mixture of 55% (v/v) acetonitrile in water containing 0.05% (v/v) formic acid was used as the mobile phase. The UV signal at 260 nm was monitored with a photodiode array detector. Authentic artemisinin (Kunming Pharmaceutical Corporation, China) was purified by re-crystallization in hexane and chloroform to be used as the standard in this experiment.

Results and discussion

Tetraploid induction and confirmation of polyploidy by cytological analysis

Although colchicine is a useful polyploidy inducing compound by inhibiting chromosome segregation during cell division, it also has a toxic effect and causes plant cell damage and death depending on the concentration for individual plant species and tissue types (Ye et al. 2010; Zhang et al. 2010; Zhang et al. 2010). In the present study, toxic effect of colchicine was also observed in leaf discs of *A. annua*. Although high plant regeneration efficiency of 60% was obtained in non-treated leaf explants after cultivation on regeneration medium for 3 weeks as previously reported (Banyai et al. 2010), colchicine-treated explants showed reduced shoot regeneration and no shoot was observed at the highest concentration tested (1%) as shown in Table 1.

Observation of metaphase chromosomes in root tip cells revealed that the plant line 500, which was selected as the plant without colchicine treatment, showed normal diploid chromosome number of $2n=2x=18$ (Figure 1A, D) as reported previously by Jesus-Gonzalez and Weathers (2003) and Wallaart et al. (1999). By using this line as a diploid control, ploidy level was examined by flow cytometric analysis for all the regenerants obtained from the colchicine-treated leaf explants. As the results, two plants, code no. 35C and 5GC, regenerated from the different explants treated with 0.1% colchicine (Table 1) showed the dominant peak at the double channel number of the control, which corresponded to the expected tetraploid value (Figure 1B–C). Tetraploidy of these two lines was also confirmed by chromosome counting of root tip cells, which showed chromosome number of $2n=4x=36$ (Figure 1E–F). Although chromosome doubling was detected in two regenerated plants derived from the explants treated with 0.1% colchicine (Table 1), more detailed studies will be needed to increase the efficiency of chromosome doubling by adjusting the appropriate conditions of the treatment such as concentration and duration of the colchicine treatment and the type of explants.

Growth characterization of tetraploid plant

It has been well known that polyploids generally show the alterations in plant morphology from its diploid counterpart (Otto and Whitton 2000; Tang et al. 2010; Ye et al. 2010). In the present study, there is no morphological difference among five diploid plants regenerated from 0.1% colchicine-treated explants. While the significant differences were found in morphological and anatomical characters such as leaf size, root size, as well as size and numbers of stomata between control diploid (code no. 500) and two tetraploids (code no 35C and 5GC) as shown in Figure 2 and Table 2. Wallaart et al. (1999) reported that the

Table 1 Effect of colchicines concentration on tetraploid induction from a diploid plant (code no. 500) of *A. annua*

Colchicine concentration (% w/v)	No of explant	Regeneration (%)	No. of shoot obtained	No. of diploid plant obtained	No. of tetraploid plant obtained
0	25	60.0	15	15	0
0.01	24	36.7	9	9	0
0.1	45	13.3	7	5	2
1.0	25	0.0	0	0	0

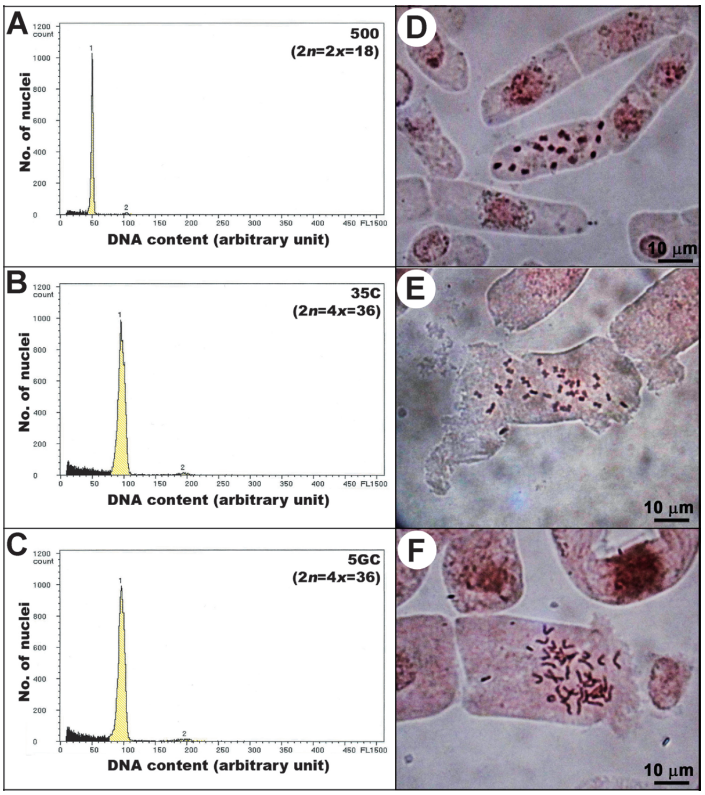

Figure 1. Flow cytometric histograms and chromosomes in root tip cells of *Artemisia annua* diploid line code no. 500 (A, D) and tetraploid lines code no. 35C (B, E) and 5GC (C, F).

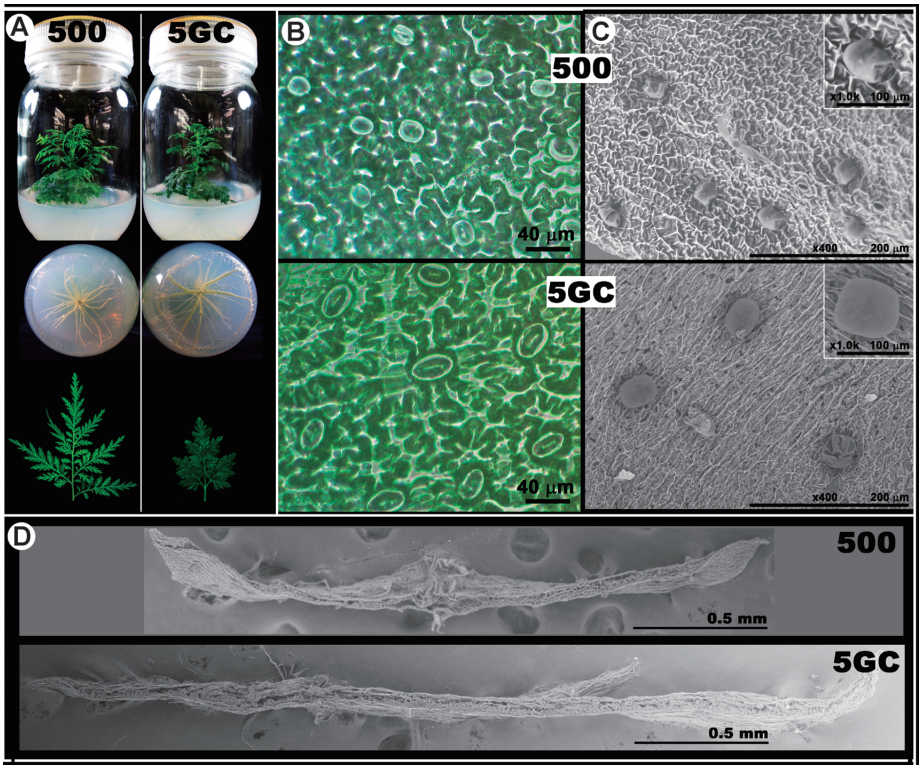

Figure 2. Comparison of morphological characters between diploid line (code no. 500) and tetraploid line (code no. 5GC) of *A. annua* under *in vitro* culture condition. (A) plant, leaf and root, (B) stomata, (C) glandular trichome, (D) leaf thickness.

Table 2 Leaf characteristics of diploid and tetraploid plants of *A. annua*

Code no.	Ploidy	Leaf length (cm)	Leaf width (cm)	Stomata density (no./mm^2)	Stomata length (μm)	Stomata width (μm)
500	2x	3.9 ± 0.4 a	3.4 ± 0.5 a	22.7 ± 2.7 a	26.2 ± 2.6 b	20.4 ± 2.1 c
35C	4x	2.9 ± 0.3 b	2.4 ± 0.2 b	12.1 ± 1.4 b	42.1 ± 3.1 a	28.3 ± 3.3 b
5GC	4x	2.8 ± 0.3 b	2.5 ± 0.2 b	20.1 ± 2.7 a	43.0 ± 3.2 a	32.7 ± 3.6 a

Different letters within the column indicate highly significant difference of mean (±SD) tested by Duncan's Multiple Range Test (DMRT) at $p \leq 0.01$. The data were analyzed from 10 replications of each treatment.

tetraploid plants were smaller than the wild-type plants but that certain individual organs like the leaves were considerably larger. In contrast, leaf size of the tetraploid lines generated in the present study was smaller than diploid although they had thicker and more dark green leaves (Figure 2A, D). Moreover, the leaf of tetraploid plants had larger stomata size, both in length and width (Figure 2B, Table 2) but the lower density of stomata per leaf area unit was found in tetraploid code no. 35C (Table 2). The obvious difference was also found in root growth. Tetraploid plant initiated thicker and longer roots than diploid line as shown in Figure 2A. Previously, Jesus-Gonzalez and Weathers (2003) succeeded to produce tetraploid hairy root of *A. annua*, but no obvious morphological differences were found and the diploid hairy root still grew better than the tetraploid line.

Recently, it was confirmed that artemisinin is biosynthesized and accumulated in GSTs of *A. annua* plants because of its high phytotoxicity to the plants themselves (Covello et al. 2007; Olsson et al. 2009). Accordingly, it is essential to confirm the density and the size of GSTs in leaves. Although the density of GSTs in the two tetraploid plants examined was less than diploid plant (Table 3), its size both in length and width was larger than those of diploid plant (Table 3, Figure 2C). This increased size was anticipated to compensate the reduced density of GSTs for the total production and accumulation of artemisinin compound.

The induction of the tetraploid whole plant of *A. annua*, from 18 to 36 chromosomes by colchicine treatment was firstly demonstrated by Wallaart et al. (1999). Although the artemisinin level in tetraploid plant was greater than that of the diploid, there is no significant difference of the overall yield of artemisinin because of low growth rate in the tetraploid line. Between two tetraploid lines obtained in the present study, code no. 35C showed slower growth rate than code no. 5GC. Moreover, GST density was also less than those of code no. 5GC. Consequently, only tetraploid plant code no. 5GC was selected to compare the growth characteristics and artemisinin production with those of corresponding diploid plant code no. 500. The *in vitro* plantlets were acclimatized, transplanted to pots, and cultured in plant growth cabinet. All of the plants were successfully grown in the cabinet but tetraploid showed significantly lower growth parameters, such as number of leaves, plant height and leaf FW, than diploid lines at 28 days after transplantation to pots (vegetative stage). They produced flowers after exposed to a short photoperiod (8 h/d) and no significant difference was found in plant flowering period between diploid and tetraploid lines (data not shown). Tetraploid line, 5GC gradually increased growth rate till flower initiation stage, at which it had the same amount of leaves FW with their diploid line. Until full blooming stage, FW of leaves in tetraploid plant became higher than those of their diploid counterpart (Table 3).

Table 3 Characteristics of glandular secretory trichomes (GSTs) in diploid and tetraploid plants of *A. annua*

Code no.	Ploidy	GSTs density (no./mm^2)	GSTs length (μm)	GSTs width (μm)
500	2x	26.7 ± 3.9 a	30.1 ± 1.9 b	25.1 ± 1.2 b
35C	4x	18.3 ± 3.9 b	46.0 ± 1.8 a	39.9 ± 2.1 a
5GC	4x	21.7 ± 3.5 ab	47.6 ± 1.6 a	42.0 ± 1.8 a

Different letters within the column indicate highly significant difference of mean (±SD) tested by Duncan's Multiple Range Test (DMRT) at $p \leq 0.01$. The data were analyzed from 10 replications of each treatment.

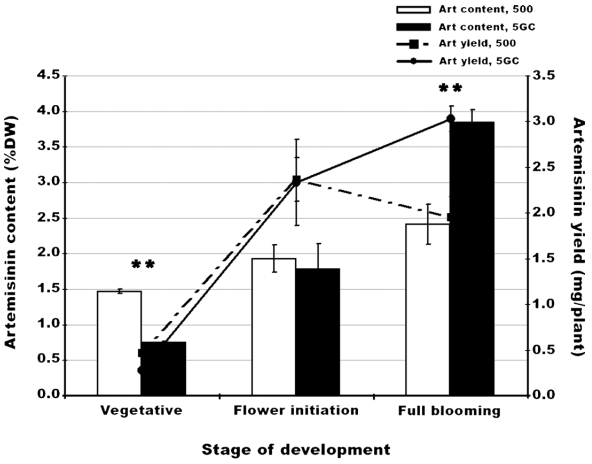

Figure 3. The levels of artemisinin content (% DW) and yield (mg plant^{-1}) at vegetative, flower initiation and at full blooming stages in diploid code no. 500 and tetraploid code no. 5GC. The data were averaged from at least 3 replicates containing three samples each and analyzed using a T-test for significant differences between diploid and tetraploid lines. Error bar means±SD. The double asterisk (**) indicates a highly significant difference at $P<0.01$.

Artemisinin production in tetraploid A. annua

It was previously demonstrated that the averaged

Table 4 Growth characteristics of diploid and tetraploid *A. annua* plants grown in plant growth cabinet

Code no. (ploidy level)	Vegetative stage			Flower initiation stage			Full blooming stage		
	No. of leaves	Plant height (cm)	Total leaves FW (g)	No. of leaves	Plant height (cm)	Total leaves FW (g)	No. of leaves	Plant height (cm)	Total leaves FW (g)
500 (2*x*)	35.5 ± 1.7	48.9 ± 1.6	7.6 ± 0.7	56.7 ± 2.9	118.9 ± 5.3	12.3 ± 0.7	38.6 ± 4.8	123.8 ± 9.3	8.1±0.4
5GC (4*x*)	30.0 ± 1.8	44.8 ± 1.9	7.4 ± 0.7	52.4 ± 3.2	106.4 ± 6.1	13.1 ± 0.6	37.8 ± 2.1	110.5 ± 5.9	9.2 ± 0.6
T-test	**	**	ns	*	**	ns	ns	*	*

The data (mean±SD) were obtained from 3 plants for both tetraploid (code 5GC) and its original diploid (code 500) lines at each developmental stage, and statistically significant difference between these two ploidy lines was analyzed with T-test.

* significant difference at $p<0.05$, ** highly significant difference at $P<0.01$, ns: non-significant difference.

artemisinin level in tetraploids was 38% higher than that of the diploids at the whole vegetative period (Wallaart et al. 1999). In the present study, however, the opposite result was obtained in our tetraploid line, code no. 5GC, which showed lower content and yield of artemisininin than the diploid about 50% at vegetative stage (28 days after transplantation). Then the artemisinin content increased thereafter in both diploid and tetraploid lines, especially drastically in tetraploid, and no significant difference in the content was found between tetraploid and diploid lines at flower initiation stage. Although the highest amount of artemisinin was produced at flower blooming stage in both lines (Figure 3), tetraploid line (3.8% DW) gave 1.6 times higher content than the diploid (2.4% DW). It was found that only 26% of artemisinin content increased in diploid during the transition from flower initiation to full bloom stage, whereas up to 111% of artemisinin content was boosted up in tetraploid line. Leaf senescence was clearly observed when flowers initiated to bloom, resulting in the drop of leaf FW in both plant lines (Table 4). This phenomenon caused the low artemisinin yield at full blooming stage in diploid plant even though the highest yield was reached at this point. Consequently, the optimum harvest time for the diploid line was flower initiation stage in which the maximum artemisinin yield at 2.3 mg plant^{-1} was obtained. Although the reduction of leaf biomass was also detected in tetraploid line, it was not so much as in diploid plant (Table 4). Moreover, the sharp increase of artemisinin content at full blooming stage was gained in tetraploid plant. Therefore, the highest artemisinin yield in tetraploid plant up to 3.0 mg plant^{-1} was detected at full blooming stage (Figure 3). These yield numbers were expected to gain more when generally cultivate in the field.

Conclusion

Although the tetraploid *A. annua* plant and hairy root were previously induced by colchicines treatment, the limitation in low production yield of artemisinin was encountered. Based on our searching, this is the first report that increase in artemisinin yield was achieved in tetraploid *A. annua* plant. Although the growth rate and artemisinin production was low at vegetative stage, the dramatic increase of these parameters in tetraploid line was detected after initiation of reproductive stage. These results clearly suggested that the harvesting period is one of the important aspects for each plant line in order to gain the maximum yield of artemisinin. Moreover, the information gained from this study could encourage the use of polyploidy induction strategy as an effective tool for producing the lines with high artemisinin yield in *A. annua*.

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