# Acclimation and Agronomic Performance of Polyploids Clones of *Artemisia annua* L. (Aklimitasi dan Kenampakan agronomi dari Klon Poliploid *Artemisia annua* L.)

**Wiguna Rahman[1], Erwin Al Hafiizh[2], Tri Muji Ermayanti[2], Deritha Ellfy Rantau[2], & Arthur A. Lelono[3]**

[1] Cibodas Botanic Garden-LIPI, [2] Research Center for Biotechnology-LIPI,
[3]Research Center for Chemistry-LIPI
**Email:** Wiguna.rahman@gmail.com

**Received**: November 2015 **Accepted:** June 2016

**ABSTRACT**

Somatic cell manipulation of *Artemisia annua* L. was conducted by induction of polyploid plants with Colchicine and Oryzalin in order to increase level of artemisinin. Polyploid plantlets were multiplied on MS medium without plant growth regulators. After acclimation processes, plants were grown in the field for agronomic performance observation. Survival rate of plantlets was recorded. Agronomic performance of plants was observed by recording height of plants, number of branches, leaf biomass, stomatal characteristics, and artemisinin content. The results showed that survival rate of the plantlets from Colchicine and Oryzalin treatments were ranging from 13.40 to 33.33% and 11.11 to 41.67%, respectively. Growth rates of plant height and plant branching were not significantly different between diploid and tetraploid plant both from Colchicine and Oryzalin treatments, except to triploid plants from Colchicine treatment. Averages of plant height from Colchicine and Oryzalin treatments were ranging from 10.0 to 220.0 cm and from 35.0 to 186.0 cm, respectively. The averages number of branches per plant of polyploid plants from Colchicine and Oryzalin treatments were ranging from 3 to 66 and from 11 to 63, respectively. Averages of dry leaves biomass between diploid and tetraploid plant from Colchicine and Oryzalin treatments were also not significantly different. They were ranging from 12 to 64 g/plant and from 11 to 62 g/plant, respectively. However, tetraploid clones have bigger size of stomata and produced more artemisinin than the diploids.

**Keywords**: *Artemisia annua* L, Colchicine, Oryzalin, Polyploids, Acclimation, Agronomic performance

**ABSTRAK**

Manipulasi sel somatic pada *Artemisia annua* L. telah dilakukan melalui induksi tanaman poliploid dengan Kolkisin dan Oryzalin sebagai salah satu cara untuk meningkatkan kadar artemisininnya. Plantlet poliploid diperbanyak dalam media MS tanpa tambahan zat pengatur tumbuh. Setelah proses aklimatisasi, tanaman ditanam di lapangan untuk diamati keragaan agronomisnya. Daya hidup plantlet saat aklimatisasi kemudian dicatat. Keragaan agronomis di lapangan diamati melalui parameter tinggi tanaman, jumlah cabang, biomassa daun, karakteristik stomata, dan kadar artemisinin. Hasil pengamatan menunjukkan bahwa tingkat kesintasan dari plantlet hasil perlakuan Kolkisin and Oryzalin berturut-turut berkisar antara 13,40–33,33% dan 11,11-41,67%. Laju pertumbuhan tinggi tanaman dan jumlah cabang tanaman diploid dan tetraploid hasil perlakuan Kolkisin dan Oryzalin tidak berbeda nyata, kecuali dibandingkan dengan tanaman triploid hasil perlakuan Kolkisin. Rata-rata tinggi tanaman hasil perlakuan Kolkisin dan Oryzalin berturut-turut berkisar antara10-220 cm and 35-186 cm. Rata-rata jumlah cabang tanaman poliploid Kolkisin dan Oryzalin berkisar antara 3-66 dan11-63 cabang/tanaman. Lebih lanjut, rata-rata bobot kering daun tanaman poliploid dari Kolkisin dan Oryzalin berkisar antara 12-64 dan 11-62 g/tanaman. Namun demikian, hasil pengamatan menunjukkan bahwa klon-klon tetraploid memiliki ukuran stomata dan kadar artemisinin yang lebih tinggi dibandingkan dengan klon-klon diploid.

**Kata Kunci**: *Artemisia annua* L, Kolkisin, Oryzalin, Poliploid, Aklimatisasi, Keragaan Agronomis.

## INTRODUCTION

*Artemisia annua* L. is a herb belongs to Sun Flower family (Compositae) producing active compound called Artemisinin. This compound is contemporary used on therapy of antimalaria and anticancer (Ivanescu & Corciova 2014). Ferreira *et al.* (2005) reported that artemisinin concentration of *A. annua* ranged from 0.1 to 0.8%. This plant is important for artemisinin production since the syntethic production is not commercially produced. This plants naturally grow in subtropical and highland area, but Gusmaini & Nurhayati (2007) elaborated that the plant was successfully cultivated in the tropics such as Indonesia. Brisibe *et al.* (2012) was also reported that *A. annua* was cultivated in the tropical lowland of Nigeria.

One of problems of *A. annua* cultivation in Indonesia is the availability of cultivars having high artemisinin content. Some cultivar of *A. annua* which are available in Indonesia have artemisinin content from 0.1 to 0.3% (Rahman *et al.* 2014). Mutated plants had high artemisinin content at 1.23% (Lestari *et al.* 2011), however there is no further report for their cultivation of this plant.

Somatic cell manipulation can be done to produce polyploid plants in order to increase the level of secondary metabolite production. There are some mitotic inhibitor agents for polyploid induction such as Colchicine, Oryzalin, Trifluralin, Amiprophos-methyl and $NO_2$ gas (Ranney, 2006). Wallart *et al.* (1999), Gonzalez & Weathers (2003) and Banyai *et al.* (2010) showed that Colchicine can induce polyploidy of *A. annua*. They also confirmed that tetraploid of *A. annua* producing higher artemisinin than the diploid. Wallart *et al.* (1999) showed that tetraploid of *A. annua* produced artemisinin 38% higher than the diploid. Gonzalez & Weathers (2003) showed that hairy root polyploid culture of *A. annua* produced artemisinin six times more than the diploid. While, Banyai *et al.* (2010) showed that tetraploid clone of *A. annua* produce artemisinin 1.5 times higher than diploid clone.

*Artemisia annua* polyploid development was also done by Hafiizh *et al.* (2013) and Ermayanti *et al*. (2014) using Colchicine and Oryzalin treatments. They had established *in vitro* triploid, tetraploid, and mixoploids plantlet of *A. annua*. The ploidy levels were tested by flow cytometric analysis and chromosome counting (Hafiizh *et al.* 2014).

One of major problem on *in vitro* plant micropropagation is acclimation process. At this stage, the abnormalities of plantlets morphology, anatomy, and physiology are repaired to *ex vitro* environment (Pospíšilová *et al.* 1999). This condition caused high percentage of plant lost or damage.

Banyai *et al*. (2010) who had success transplanted *in vitro* plantlets of *A. annua* on *ex vitro* condition reported that tetraploid plant of *A. annua* had larger size of root system, stomata, and glandular trichomes than the diploid plant. In addition, the leaf size of tetraploid was smaller but thicker than the diploid. They also found that the tetraploid plant was shorter and had less number of leaves compared to the diploid. However, Banyai *et al*. (2010) were not truly planted the plants on field condition, they grew them on controlled plant growth chamber.

This research was aimed to investigate the survival rate of planlets after acclimation processes and to determine agronomic performance of polyploid clones from *in vitro* to highland condition in order to develop new cultivar of *A. annua* with high artemisinin content.

## MATERIALS AND METHODS

Induction of polyploid plants using different concentrations of Colchicine and Oryzalin was described by Hafiizh *et al.* (2013) and Ermayanti *et al.* (2014). Shoot explants taken from two months-old of *in vitro* plantlets of *A. annua* planted on MS (Murashige & Skoog, 1962) medium without addition of plant growth regulators. The explants were then transferred on liquid MS medium containing Colchicine (0.05%, 0.1%, 0.2%, and control) or Oryzalin (7.5µM, 15 µM, 30 µM, 60 µM, 75 µM, and control).

Plants acclimation was conducted at Research Center for Biotechnology-Indonesian Institute of Sciences (LIPI) in Cibinong, West Java, in 2014. Five to seven weeks-old polyploid clones plantlets were carefully removed from culture bottles. Then, the roots were cleaned from the existing medium. Before the plantlets were transfered to small plastic polybag (5x5 cm) containing mixed of rice husk and compost (1:1), the plantlet roots were dipped into rootone powder to induce rooting. The polybags were then covered with transparant plastic, and placed under paranet shade to keep humidity high and to avoid excessive heat. After two weeks, number of survived plantlet were recorded. In total, there are 961 of plantlets which were acclimated. Plant acclimation was repeated in 2015 at two different location with different elevation, *i.e.* Cibinong (±140 m asl) and Cibodas (±1200 m asl). About 785 and 992 of planlets were acclimated at Cibinong and Cibodas, respectively. Percentage of plantlet survivorship during acclimation were compared between those two locations.

Cultivation of *A. annua* was conducted at Cibodas (±1200 m asl), West Java. Temperature and air humidity in Cibodas were range at 15-27$^{o}$C and 80-99%, respectivelly. Two to three weeks after acclimation, the survived planlets were planted on the field. They were planted on

50x100 cm distance and fertilized using NPK (40:40:40 kg/ha). Plant fertilization was conducted twice, the first were at one week after planting and the second were at one month after planting. A half dosage of N and K and full dosage of P were applied at the first time of fertilization and the remaining half dosage of N and K were applied at the second time. A surface irrigation (furrow irrigation) was used on watering the plants during dry season periods (May-September). Weeding were conducted four times with 2 weeks intervals started at two weeks after planting. Percentage of planlets which survived in the field was recorded at one month after planting. Plant growth (height and numbers of branches) were recorded every weeks during three months periods. Leaves biomass were counted at harvest time, just before plants started flowering.

Leaves from 1-2 month-old both diploid and polyploid plants were chosen for stomata evaluation. Fresh, healthy, and mature size of leaves were detached randomly at middle part of the trunk. The leaves were wrapped by moistened tissue paper and placed inside a clear plastic bag, then transferred to a cooler box to keep them moist before transported from field to lab. Leaves were placed in the refrigerator (4-6$^0$ C) to keep fresh before examination. Leaves were then cleaned from attached debris before sample preparation. About 2x5 cm$^2$ of lamina were layered by a clear nail polish at both of adaxial and abaxial surfaces and kept them in room temperature until they were dry. The nail polish containing epidermis cells were removed by cover ring the surface with clear tape then they were transferred to an object glass. Stomata were observed under microscope (LEICA DFC310 FX) at a magnification of 400 times. Length (µm), width (µm), and density of stomata were measured at ten fields of view on each sample. Stomata density defined as number of stomata per mm$^2$.

Artemisinin content were analysed based on dried leaves at harvest time. Leaves were dried on 40$^o$C for 48 hours prior to extraction. Maseration technique of methanol for 24 hour/ sample was used for extraction. The extracts were filtrated and dried under vacum condition prior to weighing. Organic solvents: n-hexane, ethyl acetate, and methanol were used for partitioning and dried before being analyzed by HPLC. Analysis of artemisinin content was conducted using Shimadzu HPLC coupled with a UV-detector at 214 nm. Isocratic condition of 1 ml/ min of acetonitril 60% was applied into reverse phase C-18 column. Artemisinin content were quantified using calibration curve of artemisinin standard at a concentration of 125, 250, 500, and 1000 ppm, respectively. Artemisinin yield were calculated by multiplied artemisinin content with mean of leaves dry mass.

Analysis of variance (ANOVA) were performed to measure the differences of plant height relatives growth rates, plant branches relative growth rates, leaves and trunk biomass, stomata characteristic (length, width, and density), artemisinin content, and artemisinin yield between ploidy levels. However, the triploid Colchicine-induced clones were not included on ANOVA of plant biomass and artemisinin due to limitation of sample number. Duncan's Multiple Range Test (DMRT) was used as further statistic analysis when ANOVA shows significant differences.

## RESULTS

### *Acclimation*

The results showed that survival rate of the plantlets from Colchicine and Oryzalin treatments and control were ranging from 13.40 to 33.33%; 11.11 to 41.67%; and 23.44% to 54.90%, respectively (Table 1). Percentage of survived plantlets from Oryzalin treatment were higher than the Colchicine, but number of survived plantlets from Colchicine were higher than Oryzalin due to more plantlets from larger Colchicine accession were acclimated. In total, the number of survived plantlets were still low that only reach under 30% of plantlets had been success on acclimation.

In 2015, number of acclimated plantlets reached up to 1777 individuals which separated in two locations. Proportion of survived plantlets was increased above the 30% when acclimation was conducted at Cibodas (Figure 1). On the other hand, rates of survived plantlets were getting lower when conducted in Cibinong which only reached 13%.

**Table 1**. Survival rate of Poly ploid *A. Annua* Plantletstreated with Colchisine and Oryzalin

| Treatment | Survival rate (%) |
|---|---|
| Control Colchicine | 23.44 |
| Colchicine 0.05% | 27.35 |
| Colchicine 0.1% | 33.33 |
| Colchicine 0.2% | 13.40 |
| Control Oryzalin | 54.90 |
| Oryzalin 7.5 µM | 34.58 |
| Oryzalin 15 µM | 11.11 |
| Oryzalin 30 µM | 33.33 |
| Oryzalin 60 µM | 41.67 |
| Oryzalin 75 µM | 14.71 |

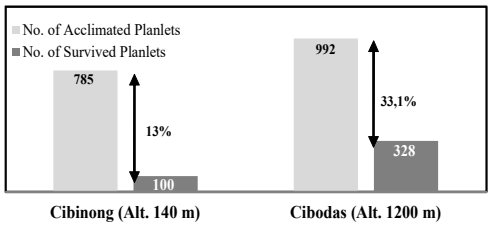


**Figure 1**. Proportion of Survived Plantlets of *A. annua* Clones during Acclimation at two Location (Cibinong and Cibodas) with different Altitude.

### *Plant Growth*

Survival rate of plantlets when transplanted in the field reached up to 65.89 % (Table 2). It was higher than survival rate during the acclimation stage. Mixoploid and tetraploid clones from colchicine treatments have higher survival rates than the other clones. However, diploid and tetraploid clones of colchicines treatment have higher number of survived plantlets which can be found in the field.

Plant height of *A. annua* in field after three months were ranging from 10-220 cm (Figure 2). The tetraploid clones from Colchicine treatment were not significantly different to diploid and mixoploid clones but significantly different to triploid clones. Plant height of diploid, mixoploid, tetraploid, and triploid clones from Colchicine treatmentafter 12 weeks in the field were ranging at10-220 cm; 25-195 cm; 70-207 cm; and 55-88 cm, respectively.While, plant height of diploid and tetraploid clones from Oryzalin treatment were ranging at 35-186 cm and 55–181 cm, respectively.

Growth of plant branching of *A. annua* clones had similar trends to plant height. It were ranging from 3 to 66 branches/plants (Figure 3). Number of plant branches of diploid, mixoploid, tetraploid, and triploid clones from Colchicine treatment were ranging at 3-66 branches; 15-59 branches; 17-58 branches, and 18-33 branches per plant, respectively. While, number of plant branches of Oryzalin diploid and tetraploid clones were ranging at 11-63 branches and 20-62 branches, respectively.

Growth rates of plant height and branching of *A. annua* clones were ranging from 0.42 to 17.5 cm/weeks (Figure 4A) and 0.25 to 6 branches/week (Figure 4B). The fastest and the slowest growth rates was found on Colchicine diploid clones. However, statistical analysis shows that the differences on plant height and plant branches growth rates among clones were significant.

**Table 2.** Survival rate of planted plantlets of *A. annua* clones in the field.

| Treatment | Ploidy level | No. Planted Planlets in the Field | No. Survived Planlets in the Fied | Percentage of Survivorship (%) |
|---|---|---|---|---|
| Colchisine | Diploid | 107 | 66 | 61.68 |
| | Mixoploid | 25 | 21 | 84 |
| | Tetraploid | 75 | 62 | 82.67 |
| | Triploid | 8 | 5 | 62.5 |
| Oryzalin | Diploid | 58 | 26 | 44,83 |
| | Tetraploid | 29 | 19 | 65.52 |
| Total | | 302 | 199 | 65.89 |

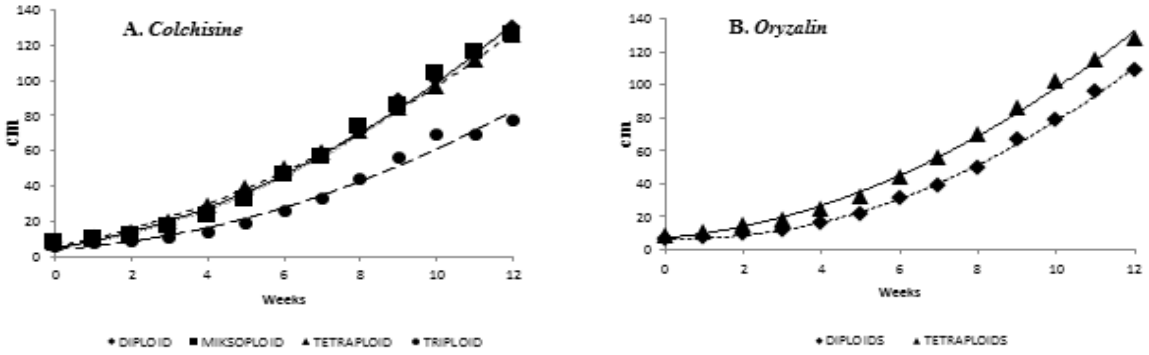


**Figure 2**. Growth of Plant Height (cm) of Diploid and Polyploid Clones of *A. annua* in Field from A.Colchicine and B.Oryzalin Treatment.

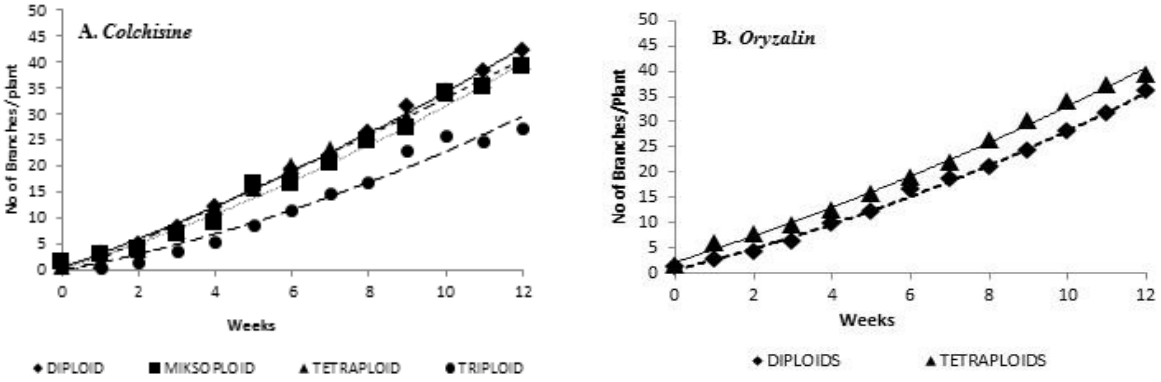


**Figure 3.** Growth of plant branches of diploid and polyploid clones of *A. annua* in field from colchicine (A) and Oryzalin (B) treatment.

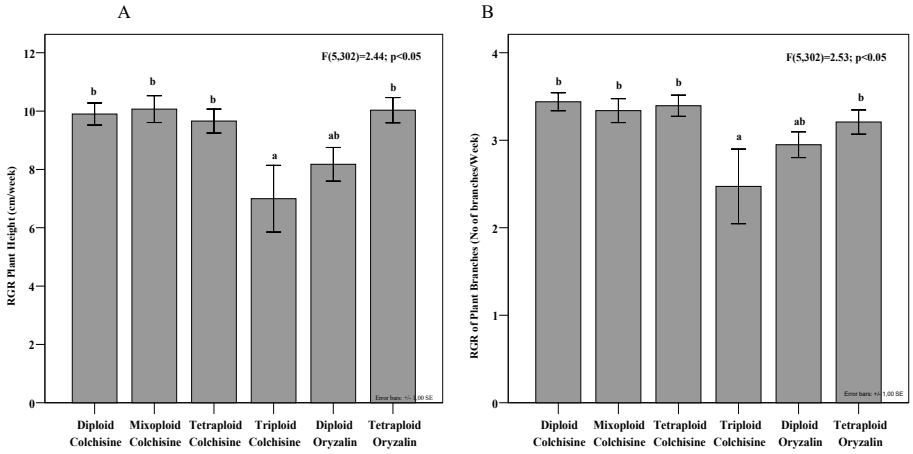


**Figure 4.** Relative growth rates (RGR) of plant height (A) and plant branches (B) of diploid and polyploid clones of *A. annua* in field from colchicine and oryzalin treatment.

### *Biomass*

There are no significant differences of leaves and trunks biomass of *A. annua* from different ploidy levels (Table 3). Leaves biomass (DW) of Colchicine diploid, tetraploids, and mixoploids clones were ranging at 22.37-95.31 g/plant, 31.99-109.88 g/plant, and 11.02-63.46 g/plant, respectively. While, leaves biomass (DW) of

Colchicine diploid, tetraploid, and mixoploids clones were ranging at 9.28-70.12 g/plant and 37.3-67.02 g/plant, respectively.

### *Stomatal Charachteristic*

Length, width, and density of stomata of *A. annua*, both after treatment with Colchicine and Oryzalin clones were significantly different among different level of ploidy (Table 4). Tetraploid clones from Oryzalin treatments have longer and wider stomata than the other clones in both of adaxial and abaxial surface. The sizes of tetraploid clones stomata were near two times higher than the diploid clones from both of Colchicine and Oryzalin treatments. More over stomata density of oryzalin tetraploid clones were almost four times lower than the colchicine diploid clones. The stomatal characteristics were not only different among the clones with different ploidy levels but also among the treatments. Stomata sizes of tetraploid clones of Colchicine treatment were smaller with one from Oryzalin treatment.

### *Artemisinin* Content

Figure 5 shows that there are significant differences on artemisinin content ($F_{(4,50)}$=2.75; $p$<0.05) and artemisinin yield ($F_{(4,50)}$=3.77;

**Table 3.** Analysis of variance of leaves and trunks biomass of different *A.annua* clones with different ploidy level based on fresh weight (FW) and dry weight (DW).

| Ploidy Level | Leaves (g) | | Trunks (g) | |
|---|---|---|---|---|
| | FW | DW | FW | DW |
| Diploid Colchicine | 211.24 ±106.6 | 57.71±25.7 | 430.88±251.44 | 131.99±70.28 |
| Diploid Oryzalin | 102.81±69.94 | 36.35±30.97 | 248.30±160.59 | 95.21±72.08 |
| Tetraploid Colchicine | 183.89±110.55 | 55.03±29.08 | 388.03±321.95 | 129.39±100.46 |
| Tetraploid Oryzalin | 188.38±41.88 | 53.39±12.31 | 433.87±156.3 | 150.92±89.55 |
| Mixoploid Colchicine | 157.88±73.76 | 35.34±15.19 | 286.64±138.11 | 87.14±41.77 |
| Sig. $F_{(4,43)}$ | ns | ns | ns | ns |

**Table 4.** Analysis of variance of stomatal length, width, and density of adaxial and abaxial leaf surfaces of diploid and polyploid *A.annua* clones. Number followed by similar word means not significantly different at P=0.05.

| Ploidy Level | Stomatal Length (µm) | Stomatal Width (µm) | Stomatal Density (No Stomata/mm$^2$) |
|---|---|---|---|
| | ---------- Leaf Adaxial Surfaces ---------- | | |
| Diploid Colchicine | 27.86a | 17.21a | 106.72c |
| Mixoploid Colchisine | 36.08b | 20.88c | 50.46a |
| Tetraploid Colchicine | 35.48b | 19.45bc | 58.63ab |
| Triploid Colchicine | 30,1a | 18.16ab | 79.18b |
| Diploid Oryzalin | 28.52a | 17.35ab | 41.54a |
| Tetraploid Oryzalin | 50.14c | 28.5d | 58.41ab |
| $F_{(5,429)}$ | 47.02 | 41.81 | 10.28 |
| $p$ | <0.001 | <0.001 | <0.001 |
| | ---------- Leaf Abaxial Surfaces ---------- | | |
| Diploid Colchicine | 26.42a | 16.15a | 196.79b |
| Mixoploid Colchisine | 34.11b | 19.62b | 108.38a |
| Tetraploid Colchicine | 35.94b | 19.66b | 119.71a |
| Triploid Colchicine | 27.99a | 16.65a | 118.77a |
| Diploid Oryzalin | 28.9a | 17.76ab | 130.45a |
| Tetraploid Oryzalin | 58.14c | 34.82c | 117.08a |
| $F_{(5,429)}$ | 74.12 | 79.27 | 21.82 |
| $p$ | <0.001 | <0.001 | <0.001 |

**Note** : ns = not significant

$p$<0.05) among *A. annua* clones with different ploidy levels and treatments. In general, tetraploid clones have higher artemisinin content (up to 0.56%) and yields (up to 297.86 mg/plant) than the others. However, tetraploid Oryzalin treated clones of *A. annua* produced more artemisinin than the Colchicine treated one. Statistically, level of artemisinin content and artemisinin yield of clones from Colchicine treatments were not significantly different due to an existence of outlier sample value of diploid Colchicine treated clone.

## DISCUSSION

Proportion of survived plantlet of *A. annua* showed having no correlation to Colchicine and Oryzalin concentrations (Tabel 1). This indicates that *ex vitro* environment during acclimation process has important role to the plantlet adaptation. During the acclimation process, the air temperature ex vitro were around 28-31$^0$C, meanwhile temperature at *in vitro* condition was set at 26-27$^o$C. This difference could be shocked to the plantlets to survive at the *ex vitro* condition. This was proved when aclimations were conducted at two different locations with different elevation (Figure 1). At the higher altitude location, plantlets survival rate was higher than the lower location. Daily air temperature at Cibodas (high elevation location) were ranged 15-27$^0$C, this condition was more similar with *in vitro* condition.

There are some factors influence planlet survival during acclimation stage *i.e.* air humidity, irradiance, $CO_2$ concentration, and absisic acid (Pospíšilová *et al.* 2007). Those factors caused changes on leaves structure, plant water status, and photosynthetic parameter. Therefore, gradual changes of environmental conditions such as air humidity and shading level from *in vitro* to *ex vitro* are needed to increase acclimation success. Pospíšilová *et al.* (2007) suggested to use anti-transpirant, ABA, and elevated $CO_2$ concentration to reduce planlet mortality at acclimation stage. In addition, growing medium is also important to increase planlet survival.

The result of plant growth were different with Wallart *et al*. (1999) and Banyai *et al*. (2010). They showed that there were significant differences on plant height between tetraploid plant and diploid plants. Tetraploid plants were smaller than that of diploid in vegetative stage, flower initiation and full blooming stage. In addition, there are lot of evidence which showed that induced tetraploid plants were shorter than the diploids such as *Lagerstromia indica, Miscanthus*, *Hebe, Dendranthema*, and citrus. On the other hand, tetraploid *A. annua* had no consistent leaf size relative to the diploid.

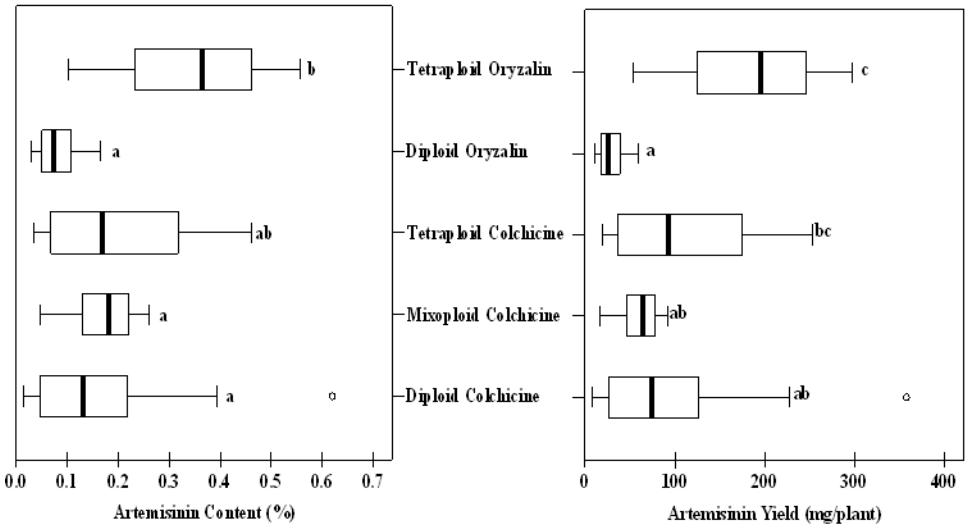


**Figure 5.** Artemisinin content (%) and Artemisinin yield (mg/plant) of diploid and polyploid clones of *A. annua*. Boxplot followed by similar word means not significantly different at α=0.05.

Wallart *et al.* (1999) stated that tetraploid plants had larger size than the diploid, while Banyai *et al.* (2010) show the contradiction. However, the result of the current experiment could be biased by limited number of clones replicates from similar accession due to survival rates of the plantlets were still low. Tetra ploid plants was confirmed by examination of stomata which was found that tetraploids of *A. annua* had bigger size compared to the diploids.

On the other hand, the result of the current experiment shows that plant biomass was concomitant to Banyai *et al*. (2010). In this experiment, the leaves biomass was harvested before plants begin to flowering. Banyai *et al*. (2010) showed that the total leaves fresh weight was not significant at vegetative and flower initiation stage but it was different at full blooming stage. Furthermore, Banyai *et al*. (2010) reported that artemisinin content and yield of tetra ploid plant was higher than the diploid at full blooming stage. Therefore, they suggested that optimum artemisinin yield for diploid plants was at flower initiation, while for tetraploid plants at full blooming stage.

## CONCLUSION

The survival rate of plantlets after acclimation processes for all accession were still low that only reached 28%. The survived plantlets from Colchicine and Oryzalin treatments were ranging from 13.40 to 33.33% and 11.11 to 41.67%, respectively. The *ex vitro* environment during acclimation process had more important role to the planlet establishment than the concentration treatment of Colchicine and Oryzalin. Moreover, there is less differences on plant growth (plant height and number of branches) and plant biomass between plants with different ploidy levels. Artemisinin level of tetraploid plants was higher than that of diploid plants. In addition, size of stomata of tetraploids was also bigger than that of diploid plants.

## REFERENCES

Banyai, W., R. Sangthong, N. Karaket, P. Inthima, M. Mii, and K. Supaibulwatana. 2010. Overproduction of artemisinin in tetraploid Artemisia annua L. *Plant Biotechnology* 27: 427–433.

Brisibe, EA., O. Udensi, PN. Chukwuraha, PM. De Magalhäes, GM. Figueira, JFS. Ferreira. 2012. Adaptation and agronomic performance of Artemisia annua L. under lowland humid tropical conditions. *Industrial Crops and Products* 39: 190-197.

Elfawal, MA., MJ. Towler, NG. Reich, D.Golenbock, PJ. Weathers, & SM. Rich. 2012. Dried Whole Plant *Artemisia annua* as an Antimalarial Therapy. PLoS One. 7(12): e52746.

Ermayanti, T.M., EA. Hafiizh, AF.Martin, & D. Rantau. 2014. Induksi tanaman poliploid *Artemisia annua* L. secara *in vitro* dengan perlakuan konsentrasi dan lama perendaman orizalin. *Prosiding Seminar Nasional XVII "Kimia dalam Pembangunan.* Yogyakarta, 19 Juni 2014. 1-8.

Ferreira, JFS., JC. Laughlin, N. Delabays, & PM. de Magalhaes. 2005. Cultivation and Genetics Of *Artemisia annua* L. for Increase Production of The Antimalarial Artemisinin. *Plant Genetic Resources* III (2): 206-229.

Gonzalez, LJ. & PJ. Weathers. 2003. Tetraploid Artemisia annua Hairy Roots Produce More Artemisinin Than Diploids. *Plant Cell Report* 21:809–813.

Gusmaini & H. Nurhayati. 2007. Potensi Pengembangan Budidaya *Artemisia annua* L. di Indonesia. *Perspektif* 6(2): 57-67.

Hafiizh, EA., TM. Ermayanti, & DE. Rantau. 2014. Tingkat ploidi *Artemisia annua* hasil perlakuan kolkisin secara *in vitro* berdasarkan metode 'squashing' dan flowsitometri. *Prosiding Seminar Nasional Biodiversitas V*. Surabaya, 6 September 2014. 330-339.

Hafiizh, EA., TM. Ermayanti, & DE. Rantau. 2013. Induksi tanaman poliploid dari kecambah *in vitroArtemisia annua* L. dengan perlakuan kolkisin.*Prosiding Seminar Nasional Kimia Terapan Indonesia* 5:117-123.

Ivanescu, B. & A. Corciova. 2014. Artemisinin in Cancer Therapy. *In* T. Aftab, J.F.S. Ferreira, M.M.A. Khan, M. Naeem

(Eds). *Artemisia annua – Pharmacology and Biotechnology*. Springer-Verlag Berlin Heidelberg.

Lestari, EG., M. Syukur, R. Purnamaningsih, R. Yunita, & R. Firdaus. 2011. Evaluation and Selection of Mutative Artemisia (*Artemisia annua* L.) According to the Altitude Variants. *HAYATI* 18(1): 16-20.

Murashige T, & F. Skoog. 1962, A revised medium for rapid growth and bioassays with tobacco tissue culture, Physiologia Plantarum 15:473-497.

Pospíšilová, J., H. Synková, D. Haisel & Š. Semorádová. 2007. Acclimation of Plantlets to Ex Vitro Conditions: Effects of Air Humidity, Irradiance, $CO_2$ Concentration and Abscisic Acid (a Review). *Acta Horticulturae* (*II*$^{nd}$ *International Symposium on Acclimatization and Establishment of Micropropagated Plants*) 748: 27-38.

Pospíšilová, J., I. Tichá, P. Kadleček, D. Haisel, & Š. Plzáková. 1999. Acclimatization of Micropropagated plants to Ex Vitro Conditions. *Biologia Plantarum* 42(4): 481-497.

Rahman,W., D. Widyatmoko, & AA. Lelono. 2014. The Effects of NPK fertilizer, Manure and Vesicular Arbuscular Mycorrhiza (VAM) on the Growth, Biomass and Artemisinin Content of *Artemisia annua* L. *Jurnal Biologi Indonesia* 10(2): 285-296.

Ranney, TG. 2006. Polyploidy: From Evolution to New Plant Development.*Combined Proceedings International Plant Propagators' Society* 56: 137-142.

Wallart, TE., N. Pras, & WJ. Quax. 1999. Seasonal Variations Of Artemisinin And Its Biosynthetic Precursors In Tetraploid Artemisia annua Plants Compared With The Wild-Type. *Planta Medica* 65: 723–728.