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Yield and artemisinin content of six polyploid accessions of *Artemisia annua* grown at medium altitude in Indonesia

Siswanto, U.[1*], Subositi, D.[2], Isnawati, A.[2] and Widiyastuti, Y.[2]

[1]Department of Crop Production, Agriculture Faculty, University Bengkulu, Indonesia; [2]Research Center for Pharmaceutical Ingredients and Traditional Medicine, Research Organization of Health, National Research and Innovation Agency, Indonesia.

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Abstract The high incidence of malaria in Indonesia has resulted in a very high dependence on malaria drugs. The effort to self-sufficiency in artemisinin raw materials has been very possible because of the suitable geographical climate for large scale cultivation of *Artemisia annua*. Artemisinin production would be efficient and economical if the content levels reach more than 0.6%. *Artemisia annua* is a short-day plant, which is a typical of sub-tropical plant. Growing this species in the tropics causes a short vegetative period and results in low levels of artemisinin. The results showed that 6 polyploid accessions of *Artemisia annua* grown in medium altitude in Indonesia demonstrated different morphological characteristics based on growth, leaf shape, branching, stem colour, and flowering characteristics. Each of evaluated accession performed growth, yield, and artemisinin content, inconsistently. Artemisinin levels of each individual sample of each accession were highly fluctuated indicating that each accession number was not genotypically stable or uniform. Further evaluation is required to determine a suitable accession to grow.

Keywords: *Artemisia annua*, Polyploid, Accession, Artemisinin, Tawangmangu

Introduction

Artemisia belongs to the Asteraceae family, consisting of nearly 200 species. *A. annua* is one of the most popular species within the genus, which has been used since immemorial time in Traditional Chinese Medicine for fever and hemorrhoids (Shahrajabian *et al*., 2020). *Artemisia* is originally from China known as Qinghao. This plant has been cultivated in many countries such as Argentina, Bulgaria, France, Brasilia and the USA (Ferreira *et al*., 2018). This plant is considered as an annual herb. It grows well in sandy or loamy soil with good drainage, pH 5.5-8.5, altitude 1000-1500 m above sea level, and rainfall from 700-1000 mm per year (Gusmaini and Nurhayati, 2015).

Artemisia is a short-day plant with a critical point of 13-hour photoperiods. This plant flowers if the photoperiod is less than 13 hours per day (Ferreira *et al.*, 1997). Its stem is erect brownish or violet brown. The

[*]**Corresponding Author:** Siswanto, U.; **Email:** usiswanto@yahoo.com

plant itself is hairless and naturally grows from 30 to 100 cm tall although in cultivation it is possible for plants to reach a height of 200 cm. The leaves of *A. annua* have a length of 3–5 cm and are divided by deep cuts into two or three small leaflets. The intensive aromatic scent of the leaves is characteristic of *Artemisia annua* (sweet wormwood) (Ferreira *et al*., 1997).

The artemisinin content in dried leaves is between 0% and 1.5% (WHO, 2005). New hybrids of *Artemisia annua* developed in Switzerland reached a leaf artemisinin content of up to 2% (Simonnet *et al.*, 2008). The small flowers have a diameter of 2–2.5 mm and are arranged in loose panicles. Their color is yellowish green. The seeds are brown achenes with a diameter of 0.6–0.8 mm. Their thousand-kernel weight (TKW) averages around 0.03 g (in comparison, wheat has a TKW of approximately 45 g). *A. annua* has a single leaf, triangular or ovoid shape, sharing double pinnate arranged alternately. Leaves are 5-12 cm long, 3-9 cm wide, pointed tip, flat base, serrated edge. *A. annua* has bisexual compound flowers arranged in the form of panicles with a yellowish crown color (Widiyastuti and Subositi, 2019).

Artemisia species produces a main metabolic seconder called artemisinin. Artemisinin is a sesquiterpene lactone with an endo-peroxide bridge. This compound is an anti-malaria because of its cytotoxic properties. This compound releases free radicals and reactive aldehydes useful for controlling *Plasmodium*. Artemisinin causes membrane damages, oxidizes proteins and fats, and inhibits the synthesis of nucleic acids in parasites to hinder its development (Graz *et al.,* 2010). Artemisinin yielded by *A. annua* varies greatly depending on the varieties. Artemisia flowers faster when grown in the tropics with low artemisinin result. In addition, *A. annua* is ecological specific with less adaptation capability. Superior clones from Vietnam had a lower artemisinin content when introduced in Brasilia and the USA (Elhag *et al*., 1992).

Yield of artemisinin correlated with location, soil, and climate of the growing area (Widiyastuti and Subositi, 2019). Growth and production period of artemisia were between 4 and 6 months. *A. annua* has usually been grown at the beginning of the year to harvest in the dry season (Omer *et al*., 2013). *A. annua* cultivated in tropical regions or at low-latitude areas affects its growth and development. The constraint of *A. annua* introduction to the tropical regions was low artemisinin content caused by earlier flowering phase due to shorter photoperiod (Widiyastuti and Subositi, 2019).

Polyploidization is an effort to produce tetraploid plants with a higher growth index and production than the wild type. A polyploid variety of *A. annua* has been tested for plant growth. In a limited field trial, it was found that polyploid varieties produced higher biomass production. But the artemisinin content was lower than their wild type in lower region. The objective of this study was to evaluate the stability of production quality of *Artemisia annua* to determine the growth capacity and adaptation of polyploid accession in higher medium altitude.

Materials and methods

The field experiments

The research was conducted at Kalisoro Tawangmangu Germplasm Collection Garden from February to October 2020, at an altitude 1,200 m above sea level. The polyploid accession tested were M1K122; M1K237; M1K299; M2K229; M2K237; M2K321. Total of 25 *A. annua* seedlings of each tested accessions were grown in trial plot (2x2,5 m), 40 x 60 cm of plant spacing and three replications. The parameters observed were plant height, stem diameter, fresh weight and dry weight of biomass, essential oil, and artemisinin content. Observation of plant height was carried out by measuring the height of the plant starting from the base of the stem to the tip of the growing point of the plant.

Artemisinin content analysis

A. annua herbs were dried in an oven at 40^0C until the water content was <12%, crushed using a blender, and filtered through a number sieve of 40 mesh. Weighed 100.0 mg of artemisia powder, placed it in a bottle with a lid, added 10.0 mL of hexane with a goiter pipette. It took 1.2 ml of liquid extract, and placed it in a centrifuge tube. Then, it centrifuged with 10,000 rpm for 5 minutes and took the supernatant to stain. Next, it prepared a standard solution by carefully weighing 1.0 mg of standard artemisinin and dissolving it with 10.0 mL of hexane (stock solution 0.1 mg/mL). And then, spotted the sample with Silica GF254 as stationary phase (10 x 20 cm), then the standard was spotted with a volume of 0.5; 1.0; 1.5; 2.0 and 2.5 mL (0.05; 0.10; 0.15; 0.20 and 0.25 mg/spot). The samples were spotted with a volume of 3 mL eluted by mixing 35 mL of hexane with 15 mL of ethyl acetate (70:30), placed it in the chromatography vessel, and added filter paper for saturation. It was waited until saturated (indicated by the wetness of the entire filter paper). Then, it was inserted thin layer chromatography (TLC) plate, waited until the mobile phase separated to reach of 8 cm of distance from the starting point of spotting. Plate was removed from the TLC vessel, then dried until all the mobile phase evaporated from the TLC plate. Visualization used anisaldehyde reagent (Acetic acid: sulfuric acid: 4-methoxybensaldehid = 50: 1: 0.5) as spray detection. Plate was heated at 105^0 C for 15 minutes and read the spot with TLC scanner 3 at wavelength of λmax 540 nm. Artemisinin appeared at Rf 0.43 with a red to purplish red color. It made a standard Curve Equation (artemisinin level vs spot area) and plotted the results of the sample readings on the standard curve equation to obtain the levels of artemisinin in the liquid extract (Y), and calculated the content level of artemisinin in the sample with the formula:

Artemisinin content = $\frac{Y \times 1000}{3 \times \text{sample weight (mg)}} \times 100\ \%$; Y= Plot value

Determination of essential oil levels

A. annua leaves were chopped to pass through the number sieve. 40 (325 mesh). Grinding was avoided to prevent the material from heating up. Prepared a distillatory apparatus, washed with petroleum ether, and dried. Weighed accurately 20 g of the sample and placed it in a round flask. Added 500 mL of distilled water to the round flask, then added a little water into the "Trap" with a pipette. Heated the flask at a distillation rate of 30 drops per minute for 6 - 7 hours. After boiling, if the volume of oil within the tube did not increase anymore, the distillation was stopped. Cooled the flask at room temperature until the oil layer was clearly visible then read the volume of oil to the nearest 0.01 ml. The essential oil content was calculated with the following formula:

Essential oil content: $\frac{\text{Oil volume read (mL)}}{\text{Sample weight (g)}} \times 100\%$
% (volume/weight)

Results

Morphological characteristics

The evaluation of agronomic performance of polyploid strain of *A. annua* was carried out by field experiment using six accessions from two levels of polyploidization treatment namely M1 (M1K299; M1K237; M1K321) and M2 (M2K122; M2K237; M2K299). All the morphological variation

The six accessions indicated different morphological characters especially for the habitus and leaf density (Figure 1). Polyploid accessions belonging to M1 treatment had larger leaves, denser leaf shapes, higher posture, and a slower flowering period compared to M2 accessions. A significant morphological appearance between two strains (M1 and M2) difference was observed in the length of the vegetative phase. The M1 strain produced individuals with a relatively longer vegetative period than the M2 strain. However, the six accessions indicated more than 50% morphological variation.

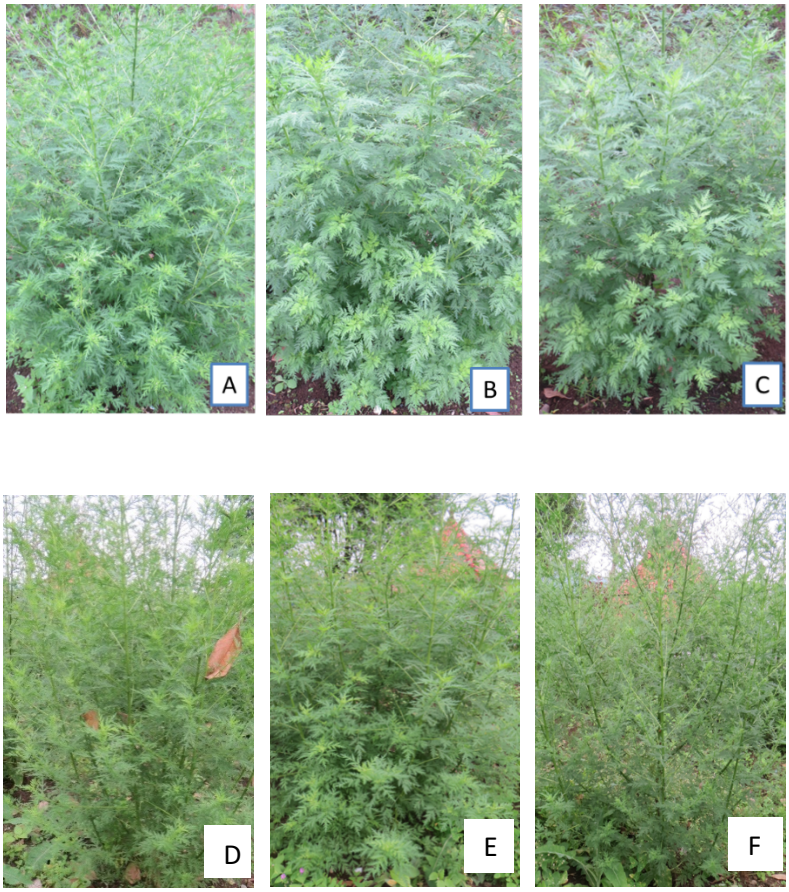

Figure 1. The morphological characteristic of six tested accessions: (A: M1K299; B: M1K237; C: M1K321; D: M2K122; E: M2K237; F: M2K299)

Growth parameters

Plant height growth was the clearest growth parameter to observe in assessing the performance of a plant field test. Plant heights from 6 accession numbers of *A. annua* tested in the field at an altitude of 1,200 m above sea level (asl) were shown in Figure 2.

Accession M2K321 reached the highest growth (163 cm), followed by M1K299 (157 cm), and M1K122 (145 cm). M1 accession had a vigorous growth compared to M2 accession. The growth of *A. annua* in the dry season showed a tendency to exponential increase in plant height entering the 6^{th} to 10^{th} week. At the beginning of the growth observed in the 4th and 6th weeks, there was no significant increase in plant height.

Stem diameter, branch number, and leaf length

Growth parameters consisted of stem diameter, branch number and leaf length. Growth of above variables of six polyploid *A. annua* accessions at an altitude of 1,200 m asl were shown in Table 1.

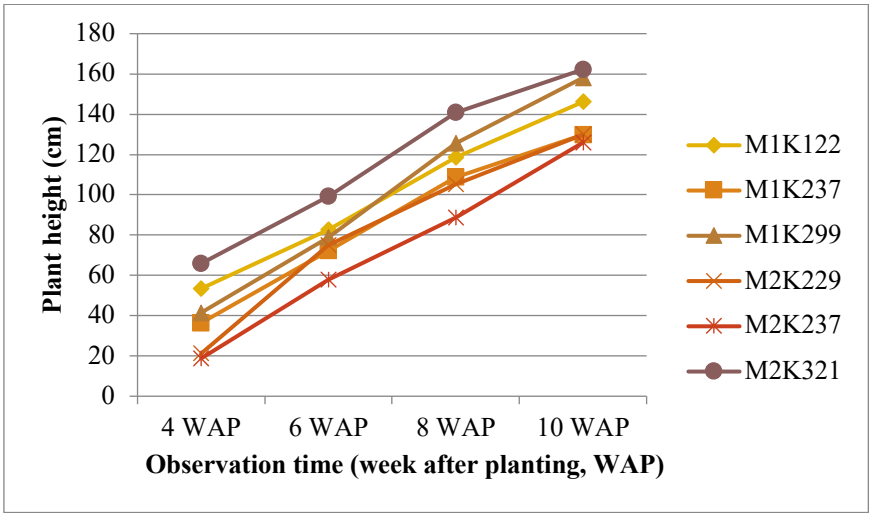

Figure 2. Plant height (cm) of 6 accession of *A. annua* observed until the 14th week after planting date

Table 1. The stem diameter (cm), number of branches and leaf length (cm) of six *A. annua* accessions observed on the 14th week after planting

	Stem diameter (cm)	Number of branches	Leaf length (cm)
M1K122	18,64[a]	44,60[a]	11,22[a]
M1K237	16,57[a]	43,40[a]	14,12[a]
M1K299	16,91[a]	45,80[a]	13,02[a]
M2K229	27,02[b]	48,20[ab]	14,48[a]
M2K237	23,88[ab]	45,40a	13,46[a]
M2K321	20,17[a]	50,80[b]	12,88[a]

Note: the same alphabetical follows the number at the same column indicated no significant different between accessions.

Result indicated that growth of stem diameter, number of branches, and leaf length of six accessions did not show a significant difference (Table 1). Accessions M2K229 and M2K237 had bigger stem diameters and M2K229 and M2K321 showed more branch number.

Biomass production

To produce superior varieties, growth and production parameters are required. The results of the biomass production of 4 *A. annua* accessions

at an altitude of 1,200 m asl were presented in Table 2. There were significant differences in all observed production parameters including fresh stem weight, fresh leaf weight and dry leaf weight. The accession M2K237 resulted in the heaviest fresh stem weight (1350.4 g), heavier fresh leaf weight (773 g), and heavier dry leaf weight (136.8 g). Accession M2K237 had a compact growth with densely branching and showed a better growth indicated by numerous branches.

Table 2. The yield of *A. annua* harvested at 16 weeks after planting

Accessions	Fresh stem weight (g)	Fresh leaves weight (g)	Dry leaves weight (g)
M1K122	593,4^{c}	360,4^{c}	77,2^{c}
M1K237	501,2^{c}	73,8^{d}	18,4^{d}
M1K299	701,4^{bc}	115,4^{d}	25,8^{d}
M2K229	1155,4^{b}	688,4^{ab}	111,6^{b}
M2K237	1350,4^{a}	773,0^{a}	138,6^{a}
M2K321	934,6^{b}	529,2^{b}	109,2^{b}

Note: the same alphabetical follows the number at the same column indicated no significant different between accessions.

Artemisinin and essential oil content

Artemisinin is the main chemical component contained in *A. annua* leaves besides the essential oil. An analysis of artemisinin compound and essential oil of six *A. annua* accession were shown in Figure 3.

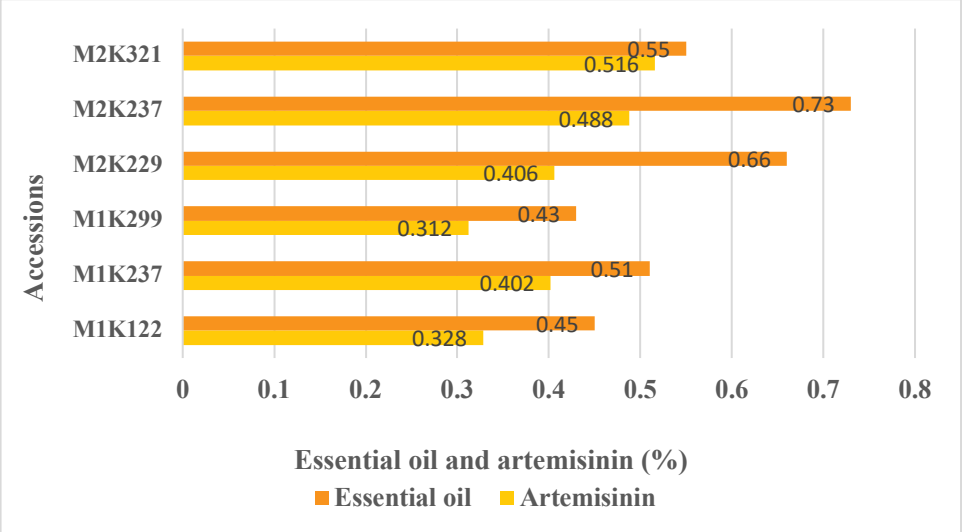

Figure 3. Artemisinin compound and essential oil content of *A. annua* grown at Tawangmangu

Figure 3 revealed that accession M2K237 produced the higher essential oil 0.73%, followed by M2K229 (0,66%), and M2K321 (0.55%). M2 accession yielded higher artemisinin than M1 accessions. There was an opposite pattern to produce essential oil and artemisinin content. Accession with higher essential oil production yielded lower artemisinin compound (M2K237), and accession with lower artemisinin production yielded higher essential oil content (M2K229).

Discussion

Artemisia annua is a naturally cross-pollinated plant with little incompatibility of flowers within the same tree. This self-incompatibility caused cross-pollination to produce viable seeds. This cross-pollination might cause high genetic variation and difficult to become homologous. Polyploidization was one of the efforts to improve genetic quality of *A. annua*. The polyploidization of *A. annua* conducted by the Biotechnology Research Center, Indonesia Institute of Science resulted in several candidate accessions for superior parental plants. In the 2020, study the stability of quality test was carried out for 6 polyploid accession numbers from M1 and M2 with 3 numbers each (Widiyastuti and Subositi, 2019).

Plant height is one of the most prominent observed and measured for plant growth parameters of *A. annua*. *A.annua* with higher habitus demonstrated more optimum growth. Six accessions of *A. annua* showed different genotype. The character of plant height growth correlated with the number of branches and leaf production. Artemisia with taller growth allows more branches and produces more leaves. These characters yielded a higher productivity. Plant growing taller produced more branches and resulted in greater biomass production. Selecting superior accessions was determined by the traits of mother plants. Similarity of habitus and morphology have led to uniformity. Accession of *A. annua* with a compact phenotype and numerous and densely branches were crucial to consider in selecting a superior clone. More branches produced more leaves and higher biomass production.

Referring to trend of plant growth per accession, there was no significant difference among accessions. Accession K321 and K122 showed consistent growth compared to other accessions. It was stated that besides being strongly influenced by genetic factors, growth was also influenced by environmental factors (Chen *et al.*, 2015). Growth was not only indicated by the increase in plant height, but also by the increasing growth in stem diameter, number of branches and leaf length. Plant height was also influenced by the growing environment, especially the availability of water, nutrients, and light. Growth was a process in plant life resulting in changes in bigger plant size and higher plant yields (Sitompul and Guritno, 1995). The increase in the size of the plant was the result of the increase in the size of the

plant organs caused by the increase in cell number and cell size (Salisbury and Ross, 1995).

Genetic variations might also influence the growth and development of the stem, leading to variations in diameter. Genetic factors promoted thicker stems, resulting in larger diameter. Further studies were required to confirm the stability of increasing the number of chromosomes from polyploid accessions grown at the medium altitude in Tawangmangu. At the same time, certain genes or genetic variations promoted branching, resulting in plants with more branches, while other genes might suppress branching, leading to fewer branches. It was stated that polyploid individuals generally grew faster and adapted easily to the environment, compared to diploid and haploid individuals. Triploid and tetraploid individuals played a role in controlling the growth of other organisms in the same habitat (Čertner *et al.*, 2017).

Plant production is the resultant of the growth process and the interaction of plants with environmental factors. The success of a plant in producing optimal production was determined by genetic traits, namely the plant's ability to grow and its interaction with the environment. Polyploid accessions derived from M1 and M2 resulted in difference biomass production of stem weight, fresh leaf weight and dry leaf weight. The actual crop production was calculated after the product was dried. *A. annua* growth was more precisely illustrated by dry weight. Fresh biomass was influenced by factors that did not reflect the actual results, such as the water content of the material. Plants grown in wet conditions were more succulent and resulted in a higher fresh weight but lighter dry weight. This was caused by high moisture content. To find an accurate production, it was necessary to measure the dry weight of the plants and their dry yield. Leaf dry weight of *A. annua* was critical to measure since the major part used as a raw material was leaf.

Accession M1K237 resulted in the highest dry yield (24%) but the lowest productivity. High yield of M1K237 accession might be the result of a larger portion of branch with fewer number and smaller size of leaves. Accessions M2K237 showed the heaviest fresh and dry leaf weight but lower dry yield (17%). This was caused by the results of a greater proportion of leaves than branches or petioles. A high dry yield reflected a high total accumulation of metabolites. Accessions with high yields of leaves and dry yields were considered in the selection of prospective clones.

A. annua is a plant producing artemisinin for malaria medicine. Artemisinin was accumulated in the trichome glands in the leaves, stems, and flowers. Physiologically, the trichome glands are damaged by the increasing age of the leaves. To avoid the destruction, it was recommended to harvest *A. annua* from the leaves on the upper 1/3 of the plant. Artemisinin is a major compound of *A. annua*. The concentration of artemisinin ranged from 0.1 to 1.1%. Based on the calculation of production efficiency, PT. Kimia Farma determined an economic content of 0.6% in simplicia (dried raw material). It

was required to increase artemisinin levels for more efficient of its cost production.

Activity of *A. annua* as an antimalarial herb is determined by 3 main chemical components found in leaves, namely artemisinin, flavonoids, and essential oils. However, because the development of preparations recommended by WHO is in the form of drugs, artemisinin is the only recommended constituent. Synthesis of artemisinin in *A. annua* resulted in an emphasis on synthesis of its essential oil. Clones with high artemisinin content contradicted with low levels of essential oil and vice versa (Delabays *et al.*, 2001).

A detailed genetic map of *A. annua* consisting of genes and markers controlling artemisinin yield has been established to produce high yielding and vigorous plants (Graham *et al.,* 2010). Identification of superior parental lines of *A. annua* with desirable traits from this genetic map has given two high yielding hybrids. Parallel line crosses and hybrids have shown consistent results for the development of enhanced *A. annua* hybrids (Townsend *et al*., 2013). Doubling the number of chromosomes resulted in new varieties of tetraploid cultivars with higher artemisinin content and these might become new elite lines (Banyai *et al.*, 2010).

Production of a whole new cultivar from various laboratories has increased artemisinin levels to about 1 to 2% on the dry weight base *(*Brisibe *et al.,* 2012*;* Delabays *et al.,* 1993*;* Ferreira *et al.,* 2005; Graham *et al*., 2010). But not all established plant lines were stable over generations (Delabays *et al.*, 2001). In this study, polyploid accessions (M1 and M2) resulted from mutations using colchicine were not able to increase artemisinin levels compared to the wild type.

Polyploid accessions of *A. annua* exhibited variations in yield and artemisinin content. While polyploidy potentially lead to increased biomass and altered secondary metabolite production, including artemisinin, the specific effects varied among different polyploid genotypes and environmental conditions. Accessions M2K229 and M2K237 had bigger stem diameter and M2K229 and M2K321 showed higher branch number. Accession of M2K237 produced the heaviest fresh stem weight (1350.4 g), heavier fresh leaf weight (773 g), and heavier dry leaf weight (136.8 g). Accession M2K237 had a compact growth with densely branching and showed a better growth. accession M2K237, M2K229 and M2K321 produced essential oil 0.73%, 0,66%, 0.55%, respectively.

M2 accession yielded higher artemisinin than M1 accession. There was an opposite pattern to produce essential oil and artemisinin content. Further research is needed to elucidate the mechanisms and optimize the use of polyploidy in improving yield and artemisinin production in *A. annua*.

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