Isolated Microspore Culture for Embryoid Production in Artemisia annua (L)

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Research Article

Keywords: Artemisia annua (L.), flower type, microspore culture, microspore stages, microspore-derived embryoid

Posted Date: November 25th, 2023

DOI: https://doi.org/10.21203/rs.3.rs-3602380/v1

Version of Record: A version of this preprint was published at Plant Cell, Tissue and Organ Culture (PCTOC) on March 20th, 2024. See the published version at https://doi.org/10.1007/s11240-024-02716-6.

Abstract

The haploidy technique is a useful tool to produce pure, fully homozygous lines in a short time. Artemisia annua (L) is a medicinal plant that produces artemisinin widely known as an anti-malarial drug. Due to its very small flower (< 3.0 mm), this study aimed to conduct microscopic observations to determine the type of flowers and their corresponding microspore development stages suitable for microspore embryogenesis and to obtain embryoids from isolated microspore cultures. Media to induce embryoid production were based on NLN medium with 13% or 17% sucrose and the addition of plant growth regulators as follows: (1) a combination of NAA and BA, designated as MCA media; and (2) a combination of 2,4-D and Kinetin, designated as MCAD media. The result indicated that due to their proportion of uninucleate and binucleate microspore stages, flowers at about to bloom (AB) and early bloom (EB) stages contained sufficient microspore stages of late uninucleate to early binucleate microspores suitable to induce embryogenesis. The production of embryoid is faster in MCA13 and MCA17 than those of MCAD13 and MCAD17. MCA13 and MCAD13 gave rise to abundant callus-like structures than those of MCA17 and MCAD17. It is indicated that the addition of 2.4-D in MCAD media inhibited the growth of the embryoid, due to the formation of a white-gluey structure covered and mixed with the embryoid.

Key message

This is the first report of successful induction of embryoids in *Artemisia annua* from isolated microspore culture, which is closely related to flower type and stage of microspore development.

Introduction

Artemisia annua (L.) belongs to the Asteraceae family, commonly known as sweet Annie or sweet annual wormwood in the United States or qinghao in China, is a shrub native to China and has long been used for medicinal purposes (Wetzstein and Janick 2019; Shen et al. 2018; Guo 2016; Willcox 2009). Malaria is one of the infectious diseases caused by *Plasmodium falciparum*. This disease is a serious threat to people globally, particularly in South-East Asia and Africa. At present, Artemisinin combination therapy (ACT) is effective and accepted as being the best malaria treatment. Artemisinin is a sesquiterpenoid/lactone compound obtained from the glandular trichomes of the annual Artemisia species. Numonov et al. (2019) found that the content of artemisinin per dry weight of 8 Artemisia species ranged from 0.07% to 0.45%. The highest content of artemisinin was observed in *A. annua* (0.45%), followed by *A. vachanica* (0.34%), while *A. dracunculus* had the lowest artemisinin content (0.07%). Low levels of artemisinin in Artemisia species are posing serious limitations in the sustainable supply of this drug and ultimately affecting the global struggle to cure malaria (Wani et al. 2021). The limited yield of artemisinin makes it a high-priced drug whose demand far outweighs its supply. Thus, this causes a growing concern, especially for people living in developing countries. One approach to achieving the required artemisinin demand is the increased cultivation of *A. annua* having high artemisinin content. Purnamaningsih et al. (2023) by using EMS successfully obtained several Artemisia

mutant lines having artemisinin content more than 0.5%. However, due to the heterozygous nature of *A. annua*, the selected mutant lines should be selected further to obtain stable generations. This unstable yield can be one of the obstacles in meeting the global artemisinin demand. Haploid technology in breeding programs has several advantages, particularly for the generation of haploid embryos that can be spontaneous or induced through chromosome doubling to be made into doubled haploid (DH) homozygous lines within a short period (Germanà et al. 2011; Ren et al. 2017). Thus, this haploid technology can be used to accelerate the development of a new cultivar in a breeding program of *A. annua*.

Such DH plants can either be produced via the culture of microspores or anthers. At present, the isolated microspore culture (IMC) technique is routinely and widely applied, especially in vegetables with small size of flowers, like in Brassicaceae and Apiaceae families (Corral-Martínez et al. 2020; Kozar et al. 2022; Shymikova et al. 2021; Romanova et al. 2023). This technique excludes the somatic cells of the donor plant, thus there is no need for an additional step of molecular testing of the regenerants to confirm homozygosity (Chiancone et al. 2015). Isolated microspore culture is more efficient because more regenerants are produced compared to those of other haploid techniques such as anther culture and unpollinated ovule culture (Qin et al. 2015). Additionally, it has been demonstrated that microspores can be easily induced to produce embryoids (Rutsgi et al. 2020).

Microspores of different species and cultivars within a species can have many different requirements for embryogenic development (Chiancone et al. 2015). Microspore embryogenesis through IMC can be affected by multiple factors such as microspore development stage; genotype, culture medium composition; microspore cell density; the effect of stress treatment, and other cultivation conditions (Dunwell, 2010; Kozar et al. 2020; Niazian and Shariatpanahi, 2020; Shumilina et al. 2020). Several studies confirmed that the stage of the male gametophyte development in a donor plant used for anther and microspore culture is the first important factor in the success of the IMC technique (Bathia et al. 2018; Dong et al. 2021; Chen et al. 2022). In addition, high sucrose has been known to increase microspore embryogenesis in the Brassica family (Dunwel and Thurling 1985; Shymikova et al. 2016), while auxin and cytokinin play positive roles in regulating cell division (Żur et al. 2015; Schaller et al. 2015).

Microspore embryogenesis research in *A. annua* is still in its infancy. To our knowledge, there is only one abstract from the publication of anther culture study in China (Wu and Tang 2012). They used the anthers of *A. annua* (L.) with mononuclear period microspores and resulted in 11 plants by the induction, formation, differentiation, and rooting of callus. Thus, there is no sufficient information on the successful use of isolated microspore culture to produce doubled haploids of *A. annua*. Therefore, this study aims to determine the type of flower based on its development stage suitable for microspore embryogenesis and compare several microspore culture media with different sucrose concentrations and plant growth regulators (PGRs) to induce the production of microspore embryoids in *Artemisia annua* (L.).

Materials And Methods

Plant material

The Microspore donor plant was a mutant line of *A. annua*, namely the T2-12-1-2 line, derived from the seed of *Artemisia annua* (L) treated with EMS from a previous study (Purnamaningsih et al. 2023). The seeds were sown in a plastic tray in the laboratory. Fertilizer was given when seedlings (15 cm) were transplanted to 10-liter plastic pots, while watering was applied 3 times a week. The seedlings of the donor plant were planted in the greenhouse until the flowering stage. Inflorescences were excised from plants and placed in a glass container filled with water when flowers in different types of stages were collected. Four types of flowers based on their developmental stages were flowers still in bud form (FB), flowers about to bloom (AB), flowers in early bloom (EB), and fully open flowers (FO). The flower collection was done in the morning.

Isolation of microspores

The flowers were surface sterilized for 20 minutes in 20% commercial bleach containing 5.25% NaOCl solution, then rinsed with sterile distilled water three times. One hundred sterile flowers from each type were freed from the stem and chopped with a scalpel after making the crosswise cut in the middle of the flower. Chopped flowers were collected into the small test tube containing 10 ml of sterile water. Then the tubes with each type of flower are shaken on a Vortex FV–2400 Micro-Spin for 3 minutes.

The crude suspension was filtered through a 40 µm nylon screen into a 50 ml centrifuge tube and then centrifuged at 1000 rpm for 5 min. The supernatant was discarded and 6 ml liquid Nitsch and Nitsch (NLN) medium with 13% sucrose was added to re-suspend the pellet. This procedure was repeated for a total of three washes. Medium for resuspend (NLN medium), contained macronutrients, full micronutrients, and the vitamins as described by Nitsch and Nitsch (1967) and modified by Lichter (1982) by adding the following ingredients per Liter: 30 mg glutathione, 800 mg L-glutamine, and 100 mg L-arginine. Microspore concentration was calculated using a hemocytometer counting chamber.

Induction of embryogenesis

The microspore density was adjusted to 12 x 10^4 cells per ml. The 0.5 ml of microspore suspension was then poured into Petri dishes (60mm x 15 mm) containing 4.0 ml of liquid induction media. Three replications each consisting of three Petri dishes were used in this study. The media used were based on NLN with 13% and 17% sucrose and the addition of PGRs as follows:

1. MCA13 : NLN medium + 13% sucrose + 0.5 mg L^{-1} NAA + 0.05 mg L^{-1} BA.
2. MCA17 : NLN medium + 17% sucrose + 0.5 mg L^{-1} NAA + 0.05 mg L^{-1} BA.
3. MCAD13 : NLN medium + 13% sucrose + 0.2 mg L^{-1} 2,4-D + 0.2 mg L^{-1} KIN.
4. MCAD17 : NLN medium + 17% sucrose + 0.2 mg L^{-1} 2,4-D + 0.2 mg L^{-1} KIN.

The pH of the medium was adjusted to 5.8. by adding 1 N of NaOH and/or HCl before autoclaving. The culture incubation was done at room temperature (26 $\pm$ 1) ^{0}C in the dark until the appearance of

embryoids, in the form of small calli or nascent embryoids, can be observed visually under the naked eye.

Observation and data analysis

Flowers and isolated microspores in cultures were observed by an Olympus CK40 Inverted Phase Microscope and Binocular Olympus SZX9 Stereo Microscope. The observation was done for the flower at different flower growth stages, the microspore development stages, the number of uninucleate microspores, the number of binucleate microspores, and the type of embryoid produced from microspores. Excel was used for statistical analysis of character means and standard deviation.

Results And Discussion

a. Relation of flower type and microspore development stages

a.1. Flower morphology of T2-12-1-2 mutant line

The flowers of *A. annua* used in this research, the T2-12-1-2 mutant line, were yellow with globelike flower heads. The size of the fully open flower was very small (2.00-3.00 mm). The flower was compositum surrounded by two series of lanceolate bracts known as phyllaries and having several individual small flowers (florets).

Four types of flowers based on their developmental stages are presented in Figure 1 as follows: (1) flowers still in bud form (FB stage) marked with long phyllaries positioned above the buds' top (Fig.1. A, B); (2) flowers about to bloom (AB stage) marked with short phyllaries positioned below the buds' top (Fig. 1. E, F); (3) flowers in early bloom (EB stage) marked with closed petal of the central disk florets and the early emergence of bifurcated stigmas of ray florets (Fig.1. I, J); and (4) fully open flower (FO) marked with the full opening of the petal of the central disk florets and longer bifurcated stigmas of ray florets (Fig.1. M, N).

The floret of T2-12-1-2 mutant line was very small in size (50-100 μm) and borne within small disk-shaped capitula (0.5-3.0 mm wide) which were organized in loose spread (Fig. 1. A, E, I). Each capitulum contained two floret types: (1) central disk florets, which were hermaphroditic (Fig.1. C, G, K), and (2) marginal ray florets, which were pistillate (Fig. 1. D, H, L). Florets were sessile and had the inferior ovary attached to a mound-shaped receptacle (red arrow in Fig. 1. C, D, G, H, K, L). Calyx was absent in the florets. Corolla comprised five united petals tubular with five recurred lobes can be seen clearly in Figure 1. (C, D, G, H, K. L). In a fully open flower, the petal was open (Fig. 1. O, P) giving the yellow color to the flower. The opening of the marginal ray florets preceded that of the central disk florets. The early emergence of the stigmatic arms of pistillate ray florets from the apical opening of the corolla could be seen in flower type AB (Fig.1. J).

After considering the insignificant size of this *A. annua* flower and the fact that only the disk florets had the anther containing microspores (Fig.1. C, G. K), thus opposite to previous anther culture research conducted in China by Wu and Tang (2012), we are convinced that for *A. annua* the isolated microspore

culture (IMC) is more suitable for producing haploid and DH rather than through the isolated anther culture (IAC), because manual dissection of anthers in compositum flower may be tedious and removal of a sufficient number of anthers from the disk florets may be difficult considering all the work shall be done under the microscope. This finding is in line with a previous report that the procedure of IMC is more timesaving and labor-saving than that of IAC due to the difficulty of excision of individual anthers from flower buds of Brassica and Apiaceae species, which also have small flowers ranging from 2.0- 4.5 mm and 1.0-1.5 mm, respectively (Shymikova et al. 2016; Dong et al. 2021; Shymikova et al. 2021)).

a.2. Microspore development stages

In this T2-12-1-2 mutant line, a single flower bud or a capitulum had 22 disk florets (Fig. 2. A) and 9 ray florets (Fig.2. B). Wetzstein et al. (2014) reported that various total numbers of florets per capitulum were observed among *A. annua* genotypes originating from open pollination of lines from several Brazilian and Chinese germplasm, and inter crosses of high-yielding lines with values ranging from 20 to 29 florets.

Disk florets of varying maturity marked with the staggered development of florets within an inflorescence were present in a single flower bud (Fig. 2. C). This is possible because the florets of *A. annua* were positioned in whorls, and the outer whorl of florets developed earlier than the inner whorls (Wetzstein et al. 2014). Therefore, microspore populations isolated from such flowers will be highly heterogeneous. In this T2-12-1-2 mutant line, one disk floret contained 5 anthers tubes (Fig.2. D, E).

Under microscope evaluation, round microspores with different stages of development from uninucleate to binucleate stages can be observed in a single disk floret within the anther tubes (Figure 2. F-K). In the uninucleate stage, the microspores contained only one nucleus. The nucleus of the uninucleate microspore was located at the center and large with dense cytoplasm in the early uninucleate and mid-uninucleate with slightly noticeable vacuoles (Fig. 2. H), but the nucleus position was off the center in the late uninucleate (Fig.2. I) stages. Mid-uninucleate was slightly bigger than early uninucleate, while late uninucleate had a bigger and more distinct vacuole than mid-uninucleate, so the nucleus was pushed to the peripheral side in the late uninucleate stage. In the binucleate stage, the microspores contained two nuclei. Early binucleate microspore showed two nuclei and cytoplasm (Fig.2. J). In this study, in most binucleate microspores, the two nuclei are similar in size and shape indicating their origin by a symmetric division. Late binucleate microspores or young pollen could be observed in a fully open flower marked with bigger and denser cytoplasm than that of early binucleate (Fig.2.K). Therefore, the development of microspores was found asynchronous in a single flower of *A. annua*.

According to García et al. (2016), microspores within the anther would gradually uptake the substances provided by the tapetum, according to their needs. The eventual metabolic differences created among microspores because of developmental asynchronies might influence their competitiveness, i.e., some microspores would be at a more advanced stage than others, possibly taking the resources first. Therefore, the selection of a flower containing microspores at the optimal stage in the donor plant is an important factor in increasing rates of microspore embryogenesis.

Generally, anthers of which microspores were at the uninucleate stage were favorable for anther culture because anther tissues could nurture the development of microspores at early stages within the anther by providing nutrients and protection against stress. In this study, similar to previous reports by Binarova et al (1997), Shariathpanahi et al (2006), and Salas et al (2012), the microspore culture represents an added physical stress during microspore isolation compared to that of anther isolation in anther culture, the late stages of pollen development might be more effective. Several studies also reported that in microspore culture of the genus *Brassica*, *Raphanus*, and *Daucus*, microspores during the late uninucleate to early binucleate stage had the potential to alter the microspores' developmental pathway from gametophytic to sporophytic (Han et al. 2014; Kozar et al. 2020; Dong et al. 2021; Shymikova et al. 2021; Romanova et al. 2023).

The proportion of uninucleate to binucleate microspores in different growth stages is presented in Table 1. From this microscope observation, it was expected that the uninucleate microspores in the FB would be dominated by the early uninucleate stage, while in AB and EB flowers would be dominated by microspores in the mid to late uninucleate stages. Thus, for binucleate stages in AB and EB flowers, the microspore population would be dominated by early binucleate microspores, while in FO flowers would be dominated by late binucleate microspores or young pollen.

Table 1 The proportion of uninucleate to binucleate microspore in the flower of T2-12-1-2 mutant line of *Artemisia annua* (L.) used for isolated microspore culture.

Flower type	The developmental stage of microspores	
	Uninucleate Stage (%)	Binucleate Stage (%)
FB	96.77 ± 8.84	3.13 ± 8.84
AB	61.22 ± 15.82	35.71 ± 15.82
EB	41.86 ± 16.62	58.14 ± 16.62
FO	20.98 ± 6.18	79.02 ± 6.18

FB= Flower bud; AB= Flower about to bloom; EB= Flower in early bloom; FO: Fully open flower

According to Mineykina et al. (2021), to select a more efficient population of microspores for the induction of embryogenesis, the individual flower containing predominantly microspores at the late uninucleate vacuolated stage and that at the early binucleate stage must be selected for each genotype because the embryoid yield would be determined by the interaction of these two factors. Generally, in anther culture research using normal and big-size flowers, the researchers used the correlation of flower bud length to microspore developmental stages to induce embryogenesis (Kumar et al. 2019; Barroso et al. 2015). In this study, for inducing microspore embryogenesis, we concluded that the only morphological marker to be used in *A. annua* with a very small composite flower (≤ 3.0 mm) is by using a certain flower type to obtain a suitable proportion of microspores at certain developmental stage.

Therefore, AB and EB flowers (Table 1) were selected for further microspore culture of *A. annua*, due to their proportion of uninucleate and binucleate microspore stages. These types of flowers (AB and EB) are visible to the naked eye and, thus can be easily differentiated (without using a microscope) from the FB and FO flower types (Fig. 1).

b. Microspore-derived embryoid (MDE)

b.1. Microspore embryogenesis in microspore culture medium

Microspore embryogenesis (ME) represents a unique system of single cell reprogramming in which a highly specialized cell, the microspore, is induced to switch its fate from gametophyte to embryo development during *in vitro* culture in plants (Prem et al. 2012; Sorriano et al. 2013; Testillano 2019). In our study, we did not sequentially track the formation of each cell structure individually. The results were obtained and analyzed by comparing the morphology of different structures recorded during the culture of microspores for 2 months, relying on literature data. The mode of development of the MDE was studied through (1). The growth of embryogenic microspores (Fig.3. A-E), (2). Development of two different forms of embryoid (Fig. 3. F-H), and (3). Microspore reprogramming (Fig. 3. I-L).

At the first embryogenic division, the microspore still exhibited the exine wall (Fig 3. A, B). The changes in the microspore population could be observed at 2 weeks after culture (WAC). The microspores divided and produced multicellular structures persisting within the microspore wall (exine) and the microspores continued to swell and burst, releasing the contents (Fig. 3. C). Under the microscope, MCA media had more visible swelled microspores than MCAD media. Then, these wall less cells were enlarged (Fig. 3. D), and some would continue to divide and form multicellular embryoid structures or primordium (Fig. 3. E). Chiancone et al. (2015) stated that when the microspores *in vitro* underwent a symmetrical division, first they formed the two-celled structures, then continued to multicellular microspores, and to large multicellular structures or proembryos. This evolution indicated that the reprogramming of the microspore and the first steps of the embryogenic pathway were achieved.

Prem et al (2012) reported that in *B. napus* under 18°C treatment, the reprogrammed microspores followed the development of two different forms of embryoid, the major pathway involving the formation of suspensor-like structures, and the minor pathway producing multicellular embryos without suspensor. In this study, although there was no temperature pre-treatment applied, the early development of embryoids could be observed under a microscope as early as 2 WAC. At 4 WAC, some microspores stopped growing after a few divisions, some formed suspensor-like structures (Fig.3.F), and the rest formed many loosely callus-like structures (Fig. 3. G).

The suspensor-like structures lagged in development, while the callus-like structures or nascent embryoids continued to grow (Fig 3. H). This situation had previously been noted for Chinese cabbage (Shumillina et al. 2015), and broccoli (Domblides et al. 2018). The size of some embryoids continued to increase, and after approximately 2 months, the microspore reprogramming and their sporophytic development were observed by the presence of multinucleated calli (Fig. 3. I, J) together with the

appearance of globular embryos. The globular embryos were pearly white, transparent, and round (Fig. 3. K). Until the end of 2.5 months of observation, the round embryos continued to grow to be elongated or were slightly oval (Fig.3. L).

b.2. Effect of high sucrose and PGRs on MDE formation

The effect of the sucrose concentration and PGRs on embryoid formation in microspore culture of T2-12-1-2 mutant lines is presented in Figure 4. Microspore-derived embryoids (MDEs) in the form of callus-like structures were visible to the naked eye in MCA medium at 6 WAC (Fig. 4. A, B, E, F) and in MCAD medium (Fig. 4. C, D, G, H) at 8 WAC. In carrots, the MDEs were visible over some time from 3-5 WAC to 6 months (Li et al. 2013; Górecka et al. 2010).

In this experiment, microspore culture medium with 13% sucrose, MCA13 (Fig.4. A, E) and MCAD13 (Fig. 4. C, G), gave rise to abundant micro calli than those of MCA17 (Fig.4. B, F) and MCAD17 (Fig.4. D, H). In an earlier study of isolated microspores from *B. napus*, Lichter (1982) found the best yields of embryoids obtained on a medium containing 12% sucrose, and some success was achieved with concentrations up to 17%. The sucrose content in the medium may be of greater importance than the concentration of macronutrients and it seems to be species-specific. A later study by Dunwell and Thurling (Dunwell and Thurling 1985) indicated that a high level of sucrose (17%) was beneficial for the induction of microspore embryogenesis of *B. napus* because the medium generates an osmotic potential similar to that of the anther homogenate, while a lower level of sucrose (13%) was important for a sustained level of microspore division. This is in line with Shymikova et al. (2021), where sucrose at relatively high concentrations might act as an osmotic stress as well as be required as an energy source for the formation of embryoid bodies.

The MCA13 and MCA17 media, an NLN-based media with 13% or 17% sucrose, supplemented with NAA and BA showed 2 weeks earlier embryogenic microspore development than the other two media containing 2,4-D and Kinetin (Fig.4. A, B, E, F). ME efficiency is affected by cell physiological status, and one component includes homeostasis of PGRs, especially a combination of auxin and cytokinin, known as key signaling molecules that control cell fate, proliferation, and differentiation (Perianez-Rodriguez et al., 2014; Zur et al. 2015). In several cases, the use of BA and NAA at low concentrations increases the effectiveness of embryoid production in microspore cultures of *Brassica* spp. (Dong et al. 2021; Niu et al. 2019; Na et al. 2011).

In the experiment with broccoli (*B. oleracea* var, *italica*), in the treatment with NAA, MDE formation decreased as the BA concentration increased above 0.5 mg L^{-1}. The MDE formation rate was much improved when 0.05 mg L^{-1} NAA and 0.01 mg L^{-1} BA were added to a half-strength NLN liquid culture medium (Na et al. 2011). Ewes et al. (2023) reported that in anther culture of *pomegranate* (*Punica gratum* L.) using anther with uninucleate pollen grains at the tetrad stage, higher concentration of NAA (1 mg L^{-1}) and BA (2 mg L^{-1}) added to modified MS medium for 30 days gave the highest value of percentage of callus induction. It was known that NAA alone would not affect embryo yields, but BAP

alone increased the MDEs formation in cabbage (*B. rapa*) as the BAP concentration increased (0, 0.01, and 0.05 mg L^{-1}) and then decreased at BAP concentration above 0.5 mg L^{-1} (Zhang et al. 2012).

The addition of 2,4-D in MCAD media gave rise to MDEs covered and mixed with something like a white-gluey structure making them difficult to observe (Fig. 4. C, D, G, H). It was suspected that 2,4-D was the cause of that phenomenon due to the higher sensitivity of microspores to 2,4-D than that of somatic cells. According to Ardebilli et al. (2011), the induction of embryogenesis in microspores needed a shorter treatment time of 2,4-D (less than one hour) compared to that of somatic cells (more than one day). Furthermore, Rodríguez-Sanz et al. (2015) stated that while microspore culture does not require exogenous 2,4-D, endogenously the phytohormone may participate in microspore reprogramming in *Brassica napus* and in vitro embryo formation.

Conclusion

The present study is the first report on an attempt to develop an isolated microspore culture for *Artemisia annua*. Flowers at about-to-bloom (AB) and early bloom (EB) stages can be selected to induce microspore embryogenesis in *A. annua* due to their suitable proportion of uninucleate and binucleate microspore stages. Those two types of flowers can be easily distinguished, and visible to the naked eye, thus making them easier to collect. MCA13 and MCA17 media gave rise to MDE earlier than those of MCAD13 and MCAD17. Microspore culture medium with 13% sucrose (MCA13 and MCAD13) gave rise to abundant MDEs in the form of callus-like structures than those of MCA17 and MCAD17. However, it is suspected that the growth of MDE in MCAD media was inhibited by 2,4-D due to the fast formation of a white-gluey structure which covered and mixed with the calli. Studies to increase the MDE rate and promote the development of obtained embryos to recover plantlets from them are now in progress.

Abbreviations

DH Doubled haploid

IMC Isolated microspore culture

PGRs Plant growth regulators

NAA Naphthaleneacetic acid

2,4-D 2,4-Dichlorophenoxyacetic acid

BA 6-benzyladenine

KIN Kinetin

EMS Ethylmethanesulphonate

NLN Nitsch and Nitsch

Declarations

Acknowledgments This work was supported by the National Research and Innovation Agency of Indonesia (BRIN) and Lembaga Pengelola Dana Pendidikan (LPDP) through RIIM project grant no. 82/II.7/HK/2022. The authors sincerely thank BBPSIP BIOGEN, Indonesian Ministry of Agriculture for their assistance in providing the facility to implement the project.

Authors Contributions

All authors contributed to the study's conception, design, and implementation of the research. Material preparation, data collection, and analysis were performed by all authors. Ragapadmi Purnamaningsih and Bambang Sapta Purwoko supervised the project. The first draft of the manuscript was written by Iswari Saraswati Dewi, Ragapadmi Purnamaningsih, and Bambang Sapta Purwoko. All authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Conflict of interest

The consent of all the authors for submission of this manuscript has been taken. The authors declare that they have no conflict of interest.

Data Availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Figures

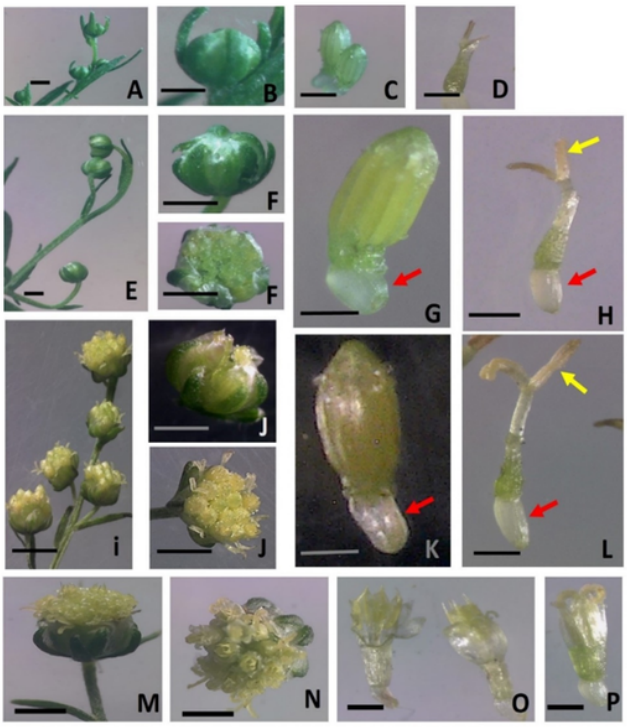

Figure 1

Performance of flower buds of T2-12-12 mutant line of *Artemisia annua* (L.). Flower in bud form (A), flower about to bloom (E), flower in early bloom (I), fully open flower (M), central disk florets (C, G, K, O), marginal ray florets (D, H, L, P). Red arrows = mound-shaped receptacle; Yellow arrows = bifurcated stigma. Bars = 1.0 mm (A, B, F, J); 1.5 mm (E, I, M, N); 50 µm (C, D, G, H, K, L, O, P)

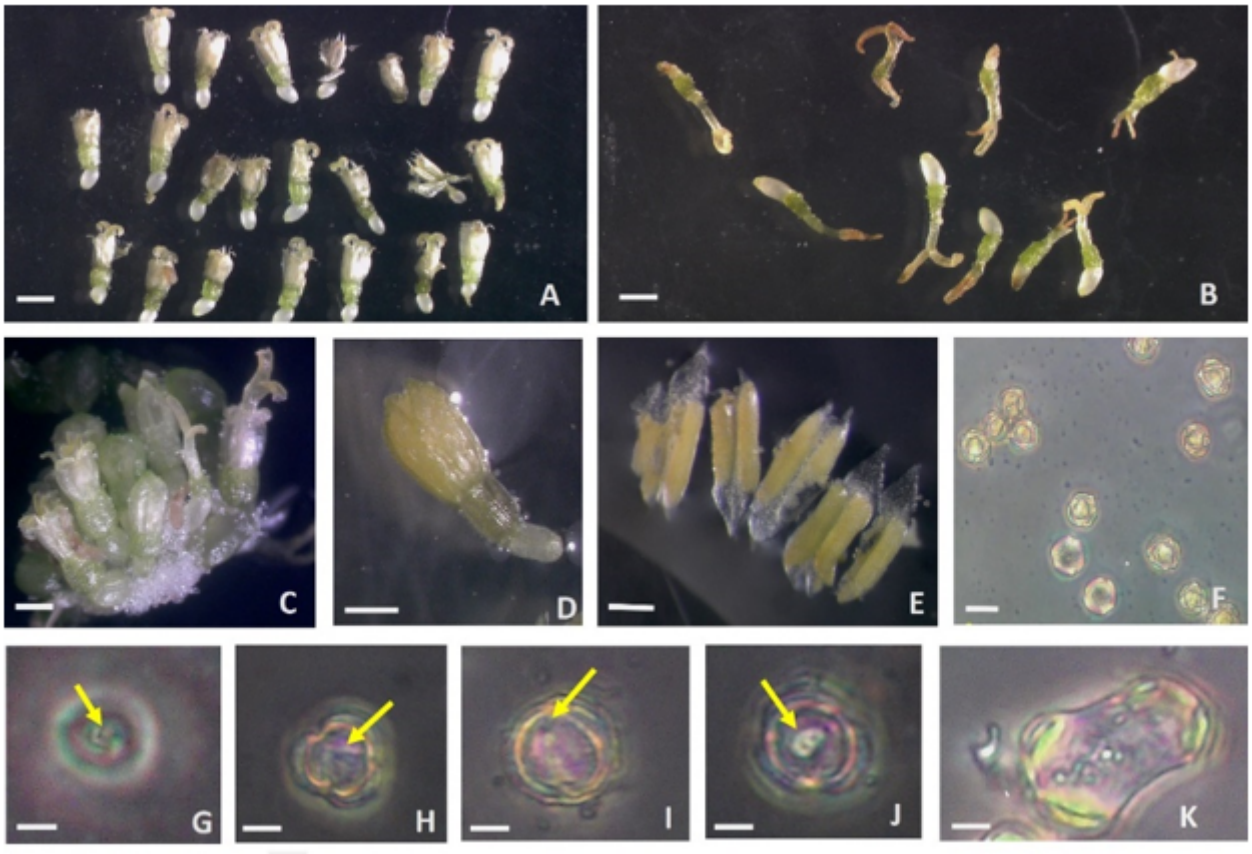

Figure 2

Florets of *A. annua* (L.). A: Disk florets; B: Ray florets; C: Florets of varying maturity with their inferior ovary attached to a mound-shaped receptacle; D: Disk florets containing anthers; E: 5 anther tubes inside a disk floret; F: Mix of different microspore development stages within the anther tube; G: early uninucleate microspore; H: Mid uninucleate microspore; I: Late uninucleate microspore; J: Early binucleate microspore; K: late binucleate microspore or young pollen. Bars: 50 µm (A-D); 10 µm (E); 5 µm (F-K)

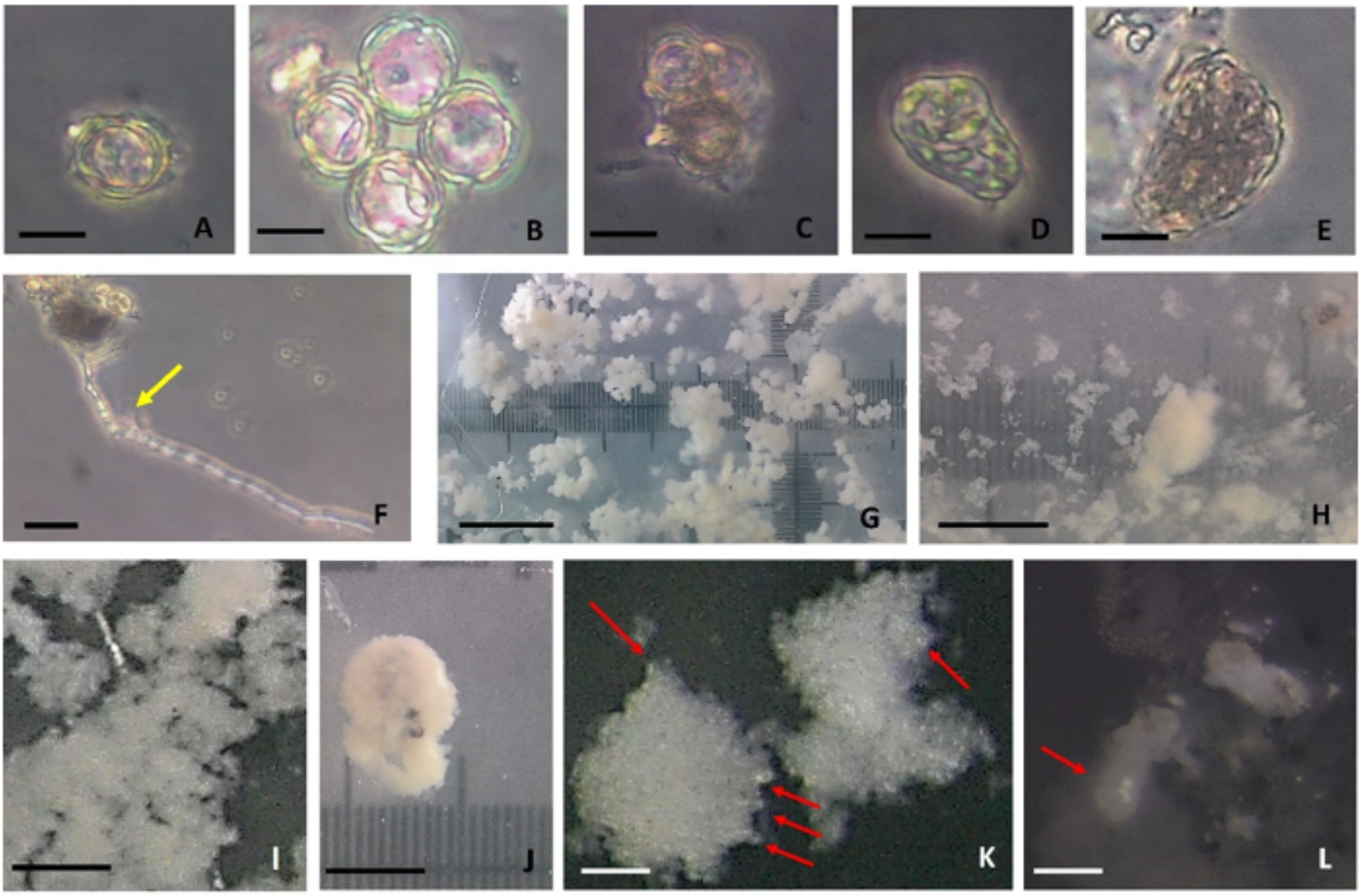

Figure 3

The development of embryoids from isolated microspores of *Artemisia annua*(L.). A-B: microspore swelled, but still exhibited the exine wall; C: microspores swelled and broke up releasing the content; D: wallless cell structure, a product from equal type of microspore division; E: multicellular embryoid structures or primordium; F: suspensor-like structure; G, H: callus-like structure; I, J: multinucleated calli; K: Multinucleate structure or proembryo; L= globular embryos at 6-7 weeks. Red arrows: globular embryos emerged from multinucleate callus-like structures; Bars: 20 µm (A-D); 50 µm (E, F); 1.0 mm (G-J).

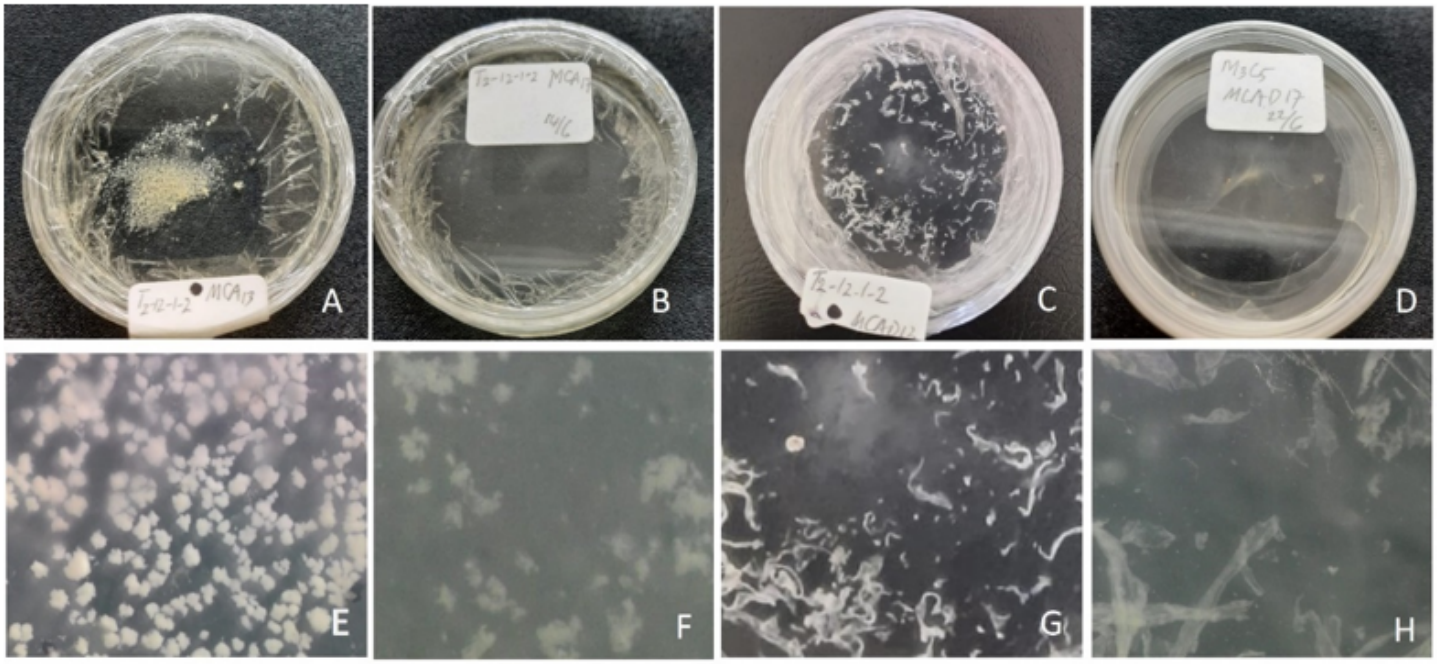

Figure 4

Microspore-derived embryoids (MDEs) in the form of callus-like structures in MCA media (6 weeks) and MCAD media at 8 weeks. MCA13 (A, E); MCA17 (B, F); IMCAD13 (C, G); MCAD17 (D, H).