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# IN VITRO CALLUS INDUCTION OF SACHA INCHI (PLUKENETIA VOLUBILIS), A PUFA-RICH PLANT

Widya Abdul Wahab[a], Jasrina Mohd Jalil[a], Norsyahira Rosle[b], Lisa Amiera Rosli[b], Tamil Chelvan Meenakshi Sundram[b,c]

[a]Department of Biotechnology, Kulliyyah of Science, International Islamic University, Kuantan, Pahang, Malaysia
[b]Department of Plant Sciences, Kulliyyah of Science, International Islamic University, Kuantan, Pahang, Malaysia.
[c]Sustainable Agriculture and Green Technology (AG-Tech) Research Unit, International Islamic University Malaysia, Jalan Sultan Ahmad Shah, Bandar Indera Mahkota, Pahang, Kuantan, 25200, Malaysia

**ABSTRACT**

*In vitro* callus induction has been established using young leaves and ovule of sacha inchi as explants. The effects of different concentrations and combinations of plant growth regulators (PGRs) on callus induction and morphology was analysed. Different treatments of 2,4-dichlorophenoxyacetic acid (2,4-D) and 6-benzylaminopurine (BAP) have influenced the callus induction under two different photoperiods, either 24 hours light or 24 hours dark conditions. The young leaves explants deemed promising in developing callus in Murashige and Skoog (MS) media supplemented with two different combinations of PGRs at different concentrations (0.1 mg $L^{-1}$ 2,4-D + 0.05 mg $L^{-1}$ BAP; and 1.0 mg $L^{-1}$ 2,4-D + 0.05 mg $L^{-1}$ BAP). Meanwhile ovule explant cultured on MS media supplemented with 0.1 mg $L^{-1}$ 2,4- D + 0.05 mg $L^{-1}$ BAP and grown under both photoperiods, 24 hours light or 24 hours dark were seen optimal in inducing callus. In addition, all the other treatments also demonstrated callus induction using ovule as explant. This is the first report attempted on using ovules as explant. The reported result shows intriguing possibilities for the utilization of sacha inchi's parts other than the seeds or leaves, as an alternative source for induction of callus culture. In future, the embryogenic callus could be developed into sacha inchi plantlets. On the other hand, the callus could also be developed into suspension cultures or batch cultures for productions of pharmaceutically important polyunsaturated fatty acids at larger scale.

**Keywords:** tissue culture; sacha peanut; 2,4-D, BAP, super food, omega

**ABSTRAK**

Kalogenesis *in vitro* telah diinduksikan menggunakan daun muda dan ovula bunga betina sacha inchi sebagai eksplan. Kesan kepekatan pengawal atur pertumbuhan (PAP) dan kombinasi PAP yang berbeza pada kadar induksi kalogenesis dan morfologi kalus telah dianalisis. Rawatan asid 2,4-diklorofenoksiasetik (2,4-D) dan 6-benzylaminopurine (BAP) pada kepekatan dan kombinasi yang berbeza telah mempengaruhi induksi kalus di bawah dua fotokala yang berbeza, sama ada 24 jam keadaan bercahaya atau 24 jam keadaan gelap. Eksplan daun muda berjaya membentuk kalus dalam media Murashige dan Skoog (MS) yang ditambah dengan dua kombinasi PAP berbeza pada kepekatan yang berbeza (0.1 mg L-1 2,4-D + 0.05 mg L-1 BAP; dan 1.0 mg L -1 2,4-D + 0.05 mg L-1 BAP). Sementara itu eksplan ovula yang dikultur di atas media MS, disuplemenkan dengan 0.1 mg L-1 2,4- D + 0.05 mg L-1 BAP di bawah kedua-dua fotokala, 24 jam terang atau 24 jam gelap didapati optimum dalam menginduksi kalus. Selain itu, menggunakan ovula sebagai eksplan semua rawatan lain dengan kepekatan dan kombinasi PAP yang berbeza juga berjaya menginduksi kalus. Kajian ini merupakan kajian pertama yang melaporkan pengunaan ovula sacha inchi sebagai eksplan. Pada masa hadapan, kalus embriogenik boleh dikembangkan menjadi anak benih sacha inchi. Selain itu, kalus juga boleh dikulturkan menjadi kultur ampaian atau kultur kelompok untuk penghasilan asid lemak tak tepu, yang penting dalam industry farmaseutikal.

**Kata Kunci**: Tisu Kultur, kekacang sacha, 2,4-D, BAP, makanan super, omega

**Corresponding author:*
***Tamil Chelvan Meenakshi Sundram***
*Kulliyyah of Science,*
*International Islamic University Malaysia*
*Email: tamil@iium.edu.my*

## 1. INTRODUCTION

Sacha inchi (*Plukenetia volubilis*), also known as "Inca peanut", "sacha peanut" or "mountain peanut" is a highly nutritious traditional food crop of the Peruvian Amazon (Krivankova et al., 2012). Regarded as the next potential "super-food" (Bueno-Borges et al., 2018), this plant from the Euphorbiaceae family has gained a lot of attention because of the excellent source of oil derived from the seeds. The cold-pressed oil has been claimed to have high concentrations of polyunsaturated fatty acids (PUFA) namely, α-linolenic acid (omega-3), and linoleic acid (omega-6) which is twice the amount found in linseed oil (Kodahl, 2020). High concentration of PUFA has been associated with prevention against diseases such as cancer, cardiovascular problems and diabetes (Carrillo et al., 2018). Besides the medicinal virtues, the leaves are also valuable as tea and vegetables. In 2014, sacha inchi oil has been declared as a safe food (GRAS) by the Food and Drug Administration (FDA) (Chasquibol et al., 2016).

Sacha inchi has been widely cultivated in Peru and Southern Colombia as part of their potential economic crops, along with several other countries such as China (Wang et al., 2014) and Vietnam (Mai et al., 2019). However, Wang et al. (2014) reported that the root of sacha inchi is susceptible to the root-knot nematode, causing damages to the plantation. Since sacha inchi is a monoecious plant mainly propagated by seeds, the chances of genetic variations are high. As this is the major problem faced by farmers, the exploration of the *in vitro* cultivation of sacha inchi is interesting and could lead to a more productive plantation.

Micropropagation is a promising method for a large size multiplication of selected clones, which requires only small space and provides lower costs compared to the conventional propagation (Moradpour et al., 2016). The variations in the plant genetics can be reduced by vegetative propagation or asexual reproduction either by cuttings (Ruiz-Solsol & Mesen, 2010; Solis et al., 2018), grafting (Cachique et al., 2011) or tissue culture (Solis et al., 2018). To our knowledge, there are only a few studies on *in vitro* technology of sacha inchi, exploring on its epicotyls, hypocotyls, apical meristem, leaf and petioles as explants (Rosle et. al. 2021; Rathore et al., 2015). Some other organs of the plant are yet to be discovered.

Herein, the present study attempts to *in vitro* micropropagate the young leaves and ovules of sacha inchi through callus induction. The outcome of the study will serve as a prelude to further exploring its totipotency, where the resultant plants might be potentially comprised of different fatty acids profile and protein content compared to the conventional plantation.

## 2. MATERIALS AND METHODS

### 2.1 Plant Materials

Leaves and ovule explants of sacha inchi were collected from the healthy mature plants grown in Glasshouse and Nursery Complex (GNC), International Islamic University Malaysia, Kuantan, Pahang, Malaysia.

### 2.2 Surface sterilization of plant tissue

The young leaves and female flowers were surface sterilized prior to inoculation on the culture media. The explants were brought into the laboratory and washed under running tap water. Five different surface sterilization methods ($S_1$-$S_5$) were conducted on the young leaves with various concentrations of sterilizing agents and time of exposure to obtain a sterile cultures (Table 1). Meanwhile, the female flowers were surface sterilized with 0.5% sodium hypochlorite (NaOCl) and Tween 20 for 10 minutes,

followed by immersion in 70% ethanol for 30 seconds prior to dissecting out the ovule (Table 1, method S6) (adopted and modified from Rosle et. al., 2021). After each treatment with the sterilizing agent, the explants were rinsed with sterile distilled water for several times in order to free them from any traces of sterilizing agents.. The culture of both explants was monitored for 15 days in the culture media.

The survival percentage of culture was calculated based on the formula:

$$\frac{Number\ of\ explants\ contaminated,browning\ or\ survived}{Total\ number\ of\ cultures\ inoculated} \times 100\%$$

- Equation 1

### 2.3 Medium preparation

Basic MS media (Murashige & Skoog, 1962) were prepared containing the macronutrients, micronutrients, vitamins, organic compound, iron source and 2g/L phytagel as a solidifying agent. The media were supplemented with 3% sucrose as a carbon source, and two different PGRs, 2,4-D and BAP at different concentrations either alone or in combination. The media were treated with 2,4-D and BAP with the concentrations of 0 and 0 mg $L^{-1}$ (treatment T1); 0.1 and 0 mg $L^{-1}$ (treatment T2); 1 and 0 mg $L^{-1}$ (treatment T3); 0.1 and 0.05 mg $L^{-1}$ (treatment T4); and 1 and 0.05 mg $L^{-1}$ (treatment T5) respectively (Table 2).

### 2.4 Explant culture

In the laminar airflow, the young leaves were cut into square pieces of about 0.5 $cm^2$ to 0.6 $cm^2$ by using sterile forceps and scalpels on a sterile paper, while ovules were carefully dissected out of the sterile female flowers on another sterile paper. The explants were then inoculated into the culture media and incubated under two different conditions; a continuous photoperiods of 24 hours light or 24 hours of dark, with an average temperature of 25 ± 2°C. Each plate consist of 4 explants with each treatment being duplicated. Data collected on the callus initiation includes the morphology, colour, percentage and size of callus, initiated at 3 weeks interval after inoculation.

## 3. RESULTS AND DISCUSSION

### 3.1 Surface sterilization of explants

Surface sterilization of female flowers to obtain the ovules was found effective by using 0.5% sodium hypochlorite (NaOCl) for 10 minutes and 70% ethanol for 30 seconds (method $S_6$, table 1) resulting in percentage of survival up to 72.2%. On the contrary, obtaining clean cultures from leaf explants was not a total success. Based on the observation and results stated in Table 1, method $S_4$ showed the highest percentage of survival at 32.5%, followed by method $S_5$ with 13.64%. Meanwhile, methods $S_1$, $S_2$ and $S_3$ recorded 0% survival of explants. The result obtained from method $S_1$ contradicts to a study conducted by Guerrero et al., (2010), which depicted that the leaf explants treated with 0.5% NaOCl for 10 mins and 70% ethanol for 30 s could obtain 100% callus induction. The ovules were dissected out of the ovary in an aseptic condition after surface sterilization to protect the soft tissues from the toxic effects of the sterilizing agent, which is in contrast to the leaves that were surface-sterilized directly with sterilizing agents.

The presence of hyphae on the culture depicted the fungi infection, while the bacterial contamination on explant was seen to be slimy and in colonies. Some of the leaf explants surface sterilized using methods $S_1$, $S_2$ and $S_3$ were not contaminated in the first week of inoculation, but they were infected

by the fungi from another explant on the same media even after 15 days. This is because microbial contamination can be latent and not able to be eliminated with surface sterilization (Singh, 2018). In method $S_4$, after a day of culture, most of the leaf explants discoloured and became pale which may be due to the overexposure of explants with 70% ethanol that is extremely phytotoxic. The ethanol used for the explant surface sterilization can dehydrate its tissue which may cause damage to tender tissue (Sameer & Nabeel, 2016). However, some of the discoloured explants survived and grew into callus. The contamination rate was observed to be higher in light condition compared to the dark condition because light is an important regulator of melanin, a condition that is optimum for the growth of fungi (Yu et al., 2013).

The causes of contamination in tissue culture varies, including ineffective surface sterilization methods, the microorganisms present in explant (endophytic) or unhygienic laboratory conditions (Ndakidemi et al., 2013). The source of plants collected could be one of the reasons contributing to the contamination. In this study, plants were grown in an open condition; thus contamination is higher as many contamination sources are present in the open field (Holvoet et al., 2015). Ndakidemi et al., (2013) mentioned that leaves grown and collected from the greenhouse depicted low levels of contamination. In addition, choosing plant material is important to avoid or reduce contamination. Young leaves are less contaminated and the tissues are potentially morphogenetic compared to the matured leaves (Ndakidemi et al., 2013). The efficiency of explants to survive with a minimal injury during surface sterilization may vary as different concentration and duration of exposure to surface sterilants was applied, depending on the types of plants or parts of plants excised. The morphological characters are one of the criteria that should be taken into consideration when applying sterilizing agents on explants, such as the tenderness and hardness of tissue (Colgecen et al., 2011; Ndakidemi et al., 2013). Sacha inchi leaf was observed to be quite tender as it could not withstand the harsh concentration of sterilizing chemicals.

### 3.2 Callus induction from leaf explant

Due to the wide contamination in the culture media, the result of callus induction on leaves could not be fully obtained. By using surface sterilization method $S_4$, the leaf explant seemed to discolour after the surface sterilization process, but callus was spotted to develop in two of the explants supplemented with 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP (Treatment T4) after a week of inoculation. The callus formed at the cut edge of the explant, as callus tends to grow on the injured part by swelling or elongation due to the excitement of tissue on explant to cover the wound (Sari et al., 2018). This continuous cell division increases as the meristematic tissues of the young leaves are exposed to auxin and cytokinin in the culture medium (Benitez-Garcia et al., 2014). The calli appeared in light green colour after three weeks of culture (Figure 1). Even though most of the explants surface sterilized using method $S_5$ were contaminated, , some survived and developed into callus when supplemented with 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP (Treatment T4). In the subsequent week, the callus was observed in greenish-brown colour and formed compact with nodular structure (Figure 2), which could be the subject of another study involving embryonic callus. The nodular structure showed the early stages of somatic embryogenesis (Moyo et al., 2009; Benítez-García et al., 2014). The results could not be compared as other treatments with the supplemented PGRs were contaminated. However, based on the observation, the

combination of 2,4-D and BAP deemed promising in inducing callus from the leaves of sacha inchi, as the combination of these PGRs will stimulate the growth and development of cell division, increase protein synthesis and affect the callus growth as well as the production of secondary metabolites (Mayerni et al., 2018).

### 3.3 Callus induction from ovule

After 3 weeks, callus induction was also observed on ovule explant on all treatments, under both 24 hours light and 24 hours dark conditions (Table 2 and Table 3). The culture on MS medium with 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP (treatment T4) developed the highest callus induction percentage (100% under 24 hours light photoperiod and 80% under 24 hours dark photoperiod), as compared to other treatments. Treatment T5 which is supplemented with 1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP induced 100% callus under 24 hours light photoperiod but only induced 75% browning callus under 24 hours dark photoperiod . Similar concentrations of 2,4-D and BAP was supplemented in the previous study, where the petioles and zygotic embryos of sacha inchi were used as explants and resulted in the best callus formation among other treatments (Pilco, 2014). This indicated that low concentration of auxin, specifically 2,4-D, when combined with low concentration of cytokinin (BAP), could potentially produce a favourable condition of callus formation.

The lowest percentage of callus was obtained in treatment T1, the control media without PGRs with 33% callus induced in 24 hours light photoperiod and 50% in 24 hours dark photoperiod. The isolated ovules in treatment T1 started to increase in size, swollen and gradually grow into callus, but the growth was too slow, with the size of 0.4 cm x 0.2 cm under 24 hours light photoperiod, and 0.3 cm x 0.2 m under 24 hours dark photoperiod. Such finding contradicts to a study of the same species by Pilco (2014), depicting that there was no callus development in media without PGRs when using petioles as explant. However the zygotic embryos tended to form callus with slow growth. Another study revealed that callus tends to grow from the hypocotyls of sacha inchi on the medium without PGRs, with the lowest shoot formation rate when compared to the other callus with PGRs when grown into shoots (Restrepo-Osorio et al., 2020).

Callus was seen to develop from the ovule inoculated in the MS medium supplemented with only 2,4-D in Treatment T2 (0.1 mg $L^{-1}$ 2,4-D) and Treatment T3 (1 mg $L^{-1}$ 2,4-D), but lower callogenic capacity compared to the treatments supplemented with both auxin and cytokinin. This shows that 2,4-D alone can induce callogenesis from sacha inchi ovule even at low concentrations without the presence of cytokinin. However, through our observation, the combination of 2,4-D and BAP produced a higher callus induction compared to the culture supplemented with only 2,4-D. 2,4-D triggers the elongation of cell by the loosening of cell wall, but cell division could not occur rapidly without cytokinin. Hence, it is said that the combination of both auxin and cytokinin increase the size of callus (Sari et al., 2018).

The texture of callus was seen to be either compact or friable. The friable callus observed in Figure 3 may be due to the action of 2,4-D which stimulates the cell elongation. Friable calli contain much water as the cell wall is not rigid and does not lignify yet, making it easy for the group of cells to be separated from the others (Sari et al., 2018). The explants cultured on MS medium supplemented with PGRs and incubated under the 24 hours light photoperiod tend to form green coloured callus (Figure 3). In contrast explant cultured and grown under 24

hours dark photoperiod produced creamy yellow, yellowish (Figure 4) and brown callus. The differences may be related with the presence of chlorophyll in tissue. The green colour appeared due to more chlorophyll content in the callus as photosynthesis took place under the white light (Siddique & Islam, 2015). The variation in callus colour might also be influenced by the diverse types and concentrations of PGRs, and type of explants (Sari et al., 2018). The callus formed in the culture without PGRs under 24 hours light photoperiod appeared to change from green colour to creamy yellow in two weeks interval, which may be caused by the degradation of chlorophyll (Sari et al., 2018).

The growth of callus under 24 hours light photoperiod is more promising compared to 24 hours dark photoperiod, based on the higher percentage and slightly bigger size of callus induced from the culture (Table 2 and Table 3). Light plays an important role in the signalling and regulatory mechanism involved in the metabolic and developmental processes of the plant, as well as being the primary source of energy (Eckstein et al., 2012). The callus induced might carry photosynthetic pigments under the light condition, which made them autotrophic thus producing carbohydrates and other metabolites. This correlated with Yildiz et al., (2012), who mentioned that chlorophyll content is the photosynthetic capacity indicator that represents the metabolic activity of the tissue. Hence, high chlorophyll induced a high photosynthetic activity which increases the production and morphology of callus.

Based on the observation, both young leaves and ovules were more likely to develop callus in Treatment T4, MS supplemented with 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP. This finding proved that the intermediate ratio of auxin and cytokinin could produce better callus through its counter and balance in cell division and elongation compared to the single use of auxin.

## 4. CONCLUSION

In conclusion, the highest callus initiation was obtained from a combination of 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP, depicting that the combination of these PGRs recorded the best response on the ovules of sacha inchi, and the callus formed successfully on the young leaves despite the contamination. To our knowledge, this is the first report on the micropropagation of sacha inchi using ovule. Even though the age of callus was still young, the differences in callus percentage, morphology and sizes indicated the efficiency of callus induction based on PGRs supplemented on the culture media. The described protocol provides a wider perspective in tissue culture system via callogenesis of sacha inchi which can be further used as a prelude in comparing the potential of the resultant plants with the traditional breeding in terms of its fatty acids and protein contents.

**Conflict of interest**: Authors declare no conflict of interest


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**Table 1.** Percentage of survival of explants by different concentrations of sterilizing agents and time exposure

| Surface sterilization method | Part of explant | Sodium hypochlorite + Tween 20 | 70% Ethanol | Percentage of survival (%) |
|---|---|---|---|---|
| $S_1$ | Leaf | 0.5 %, 8 mins | 30 secs | 0 |
| $S_2$ | Leaf | 2.0 %, 10 mins | 30 secs | 0 |
| $S_3$ | Leaf | 5.0 %, 10 mins | 30 secs | 0 |
| $S_4$ | Leaf | 1.05 %, 5 mins | 5 mins | 32.5 |
| $S_5$ | Leaf | 1.0 %, 15 mins | 60 secs | 13.6 |
| $S_6$ | Ovule | 0.5 %, 10 mins | 30 secs | 72.2 |

**Table 2.** Effects of different concentrations and combinations of PGR on ovules of Sacha inchiunder 24 hours light photoperiod after 3 weeks

| Treatment | 2,4-D (mg $L^{-1}$) | BAP (mg $L^{-1}$ ) | Callus induction (%) | Color | Texture | Size of callus (cm) |
|---|---|---|---|---|---|---|
| T1 | 0 | 0 | 33 | White cream | Compact | 0.4 x 0.2 |
| T2 | 0.1 | 0 | 6 | Light green | Friable | 0.5 x 0.5 |
| T3 | 1 | 0 | 75 | Light green | Friable | 0.3 x 0.3 |
| T4 | 0.1 | 0.05 | 100 | Light green | Friable | 0.6 x 0.7 |
| T5 | 1 | 0.05 | 100 | Light green | Friable | 0.6 x 0.4 |

**Table 3.** Effects of different concentrations and combinations of PGR on ovules of Sacha inchi under 24 hours dark photoperiod after 3 week.

| Treatment | 2,4-D (mg $L^{-1}$) | BAP (mg $L^{-1}$) | Callus induction (%) | Color | Texture | Size of callus (cm) |
|---|---|---|---|---|---|---|
| T1 | 0 | 0 | 50 | Creamy yellow | Compact | 0.3 x 0.2 |
| T2 | 0.1 | 0 | 20 | Creamy yellow | Small, compact | 0.2 x 0.2 |
| T3 | 1 | 0 | 50 | Creamy yellow | Compact | 0.4 x 0.4 |
| T4 | 0.1 | 0.05 | 80 | Yellowish | Friable | 0.6 x 0.5 |
| T5 | 1 | 0.05 | 75 | Brown | Compact | 0.5 x 0.3 |


**Figure 1:** Light green callus induced from leaf explants cultured on MS medium supplemented with 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP (treatment T4) after 3 weeks of culturing. The explant were surface sterilized using $S_4$ surface sterilization method.

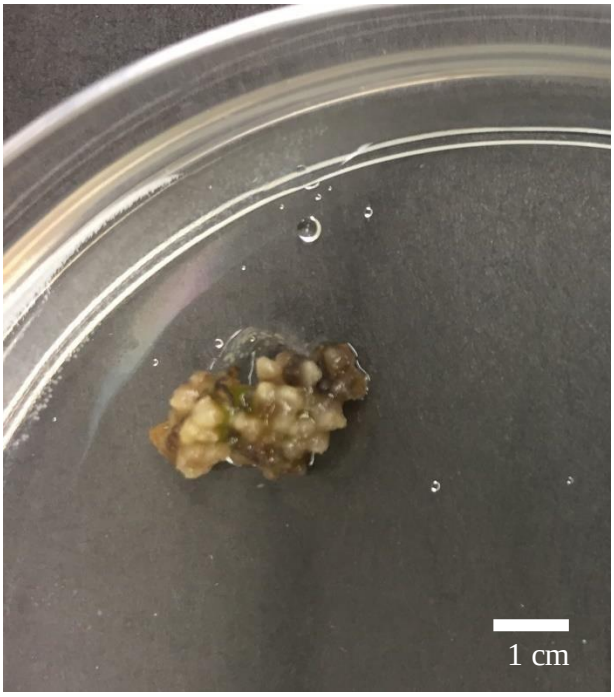


**Figure 2:** Nodular callus induced from leaf explants cultured on MS supplemented with 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP (treatment T4) after 3 weeks of culture. The explant were surface sterilized using $S_5$ surface sterilization method.


**Figure 3:** Green friable callus induced from ovule explant cultured on MS medium supplemented 0.1 mg $L^{-1}$ 2,4-D and 0.05 mg $L^{-1}$ BAP (treatment T4) under 24 hours light photoperiod after 3 weeks of *in vitro* culture.

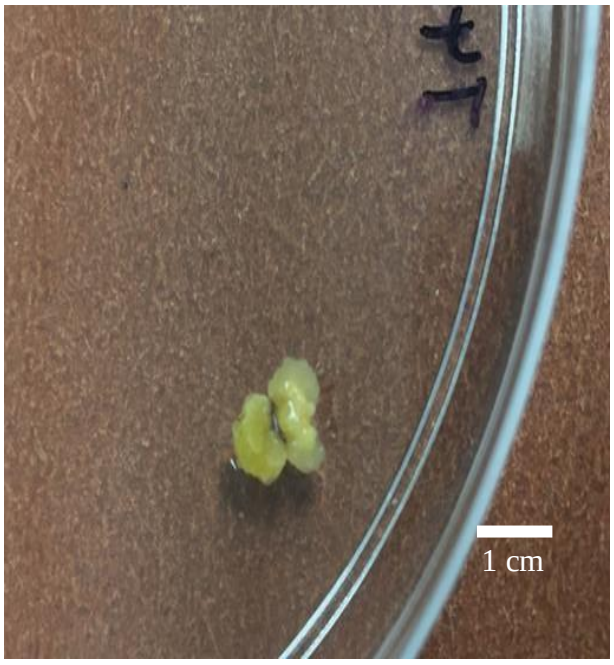


**Figure 4:** Yellowish friable callus induced from ovule explant cultured on MS medium supplemented 0.1 mg L-1 2,4-D and 0.05 mg L-1 BAP (treatment T4) under 24 hours dark photoperiod after 3 weeks of *in vitro* culture.