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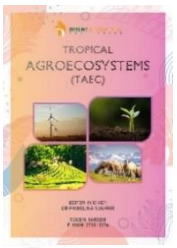

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RESEARCH ARTICLE

OPTIMISATION OF CULTURE CONDITION FOR SACHA INCHI (*Plukenetia volubilis*) CALLUS INDUCTION

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ABSTRACT

Plukenetia volubilis or commonly known as sacha inchi is reported to produce wide range of health-promoting bioactive metabolites. These metabolites functions as supplements in eradicating various types of diseases. Sacha inchi has large edible seeds that are rich in phenolic content, minerals and essential fatty acid, such as omega 3 (ω-3), omega 6 (ω-6), omega 7 (ω-7), and omega 9 (ω-9). *In vitro* cultures could serve as alternative in producing many essential sacha inchi bioactive compounds. As an initial step towards initiating *in vitro* culture, the effect of 2,4-D and TDZ on inducing callus cultures were investigated. Two different explants, sacha inchi leaf and male flower were used in this study. Surface sterilization of sacha inchi was first optimized to overcome culture contamination. The most effective surface sterilization is by using 70% ethanol (30 seconds) and 0.5% sodium hypochlorite (8 minutes), which resulted in 82.5% and 95% survival rate for leaf and flower explants respectively. Next, for calli induction the explants were cultured on MS medium supplemented with different concentrations of 2,4-D and TDZ, either alone or in combination and grown at 24 hours dark photoperiods. The morphology and size of callus were observed. The results obtained from the experiment varied depending on the treatments, producing either friable or compact calli of creamy white, pure white or brownish colour. For both, leaf and male flower explants, MS medium supplemented with 3% (w/v) sucrose in combination with 1.0 mg/L 2,4-D and 0.005 mg/L TDZ recorded the best response in term of callus size, forming friable creamy white callus.

KEYWORDS

Sacha inchi, callus, *in vitro* cultures, *Plukenetia volubilis*.

1. INTRODUCTION

Plukenetia volubilis, or commonly known as sacha inchi belongs to Euphorbiaceae family. This plant is native to rain forest of Andean region of South America (Kumar et al., 2017). It has triangular-ovate leaves with many male flowers and single female flower in a thyrse. Meanwhile the fruits are star shaped with four- to six-points, with a seed inside each star points (Carrillo et al., 2018). Sacha inchi has been cultivated since 16th century in Peru and traditionally consumed due to its nutritional value. In recent years, attention is given to this plant as a new oilseed crop and is included into agricultural sector of Amazon (Cachique et al., 2011). Extract of this plant was found to contain wide range of health-promoting bioactive metabolites, such as various phenolic compounds, tocopherol, phytosterol and essential fatty acids, such as ω-3 and ω-6 which functions as supplements in eradicating various types of diseases (Chirinos et al., 2013; Srichamnong et al., 2018). Due to the health-promoting benefits, sacha inchi seed oil is now marketed as a dietary supplement and as edible oil.

However, conventional propagation of sacha inchi is hindered by susceptibility of the plants towards nematodes, poor seed viability and delayed rooting of seedling. Moreover, the extraction process of compounds from seeds are also lengthy. Thus, *in vitro* regeneration could be a reliable alternative for mass propagation of sacha inchi as well as to be used as source for bioactive compounds production within a shorter time period (Cai et al., 2011). *In vitro* propagation of sacha inchi has been initiated by using specific types and concentrations of plant growth regulators (Patthanajuck and Bunnag, 2017). The different concentrations or combinations of plant growth regulators can give effects on *in vitro* regeneration when applied on various explants such as stem, leaf, epicotyl, hypocotyl and petiole (Mubashar et al., 2015; Rathore et al., 2015).

Callus is dedifferentiated plant cells induced on media usually containing relatively high auxin concentrations or in combination of auxin and cytokinin under *in vitro* conditions. Callus initiation of sacha inchi are induced by adding specific plant growth regulators (PGRs) in basal MS medium. To date there have been only few studies on *in vitro* propagation of sacha inchi, made to cater the problems occurring through conventional methods (Patthanajuck and Bunnag, 2017). However, sacha inchi leaves have been used as explants in inducing callus. Sacha inchi leaves promote callogenesis when cultured on MS media treated with 2.0 mg/L of 2.4-D and TDZ ranging from 0.0 to 0.1 mg/L with 100% rate of callogenesis activity (Guerrero-Abad et al., 2010). It is found that the explants under treatment of 2.0 mg/L 2.4-D and 0.005 mg/L TDZ shows formation of friable callus with the highest rate of multiplication for embryogenic callus that passed the first globular stage differentiation. To the best of our knowledge, there is no study yet done on callus induction using sacha inchi male flower as explants. Hence, in present study apart from using leaf as

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explant for initiating callus culture, we also aimed to find out the proficient callus initiation protocol by using sacha inchi male flower as explant.

2. Materials and Methods

2.1 Plant material

Immature leaves and male flower buds of sacha inchi plant to be used as explants were collected from sacha inchi plants in Glasshouse and Nursery Complex (GNC), IIUM Kuantan Campus.

2.2 Surface sterilization procedure

Upon inoculation process, explants were surface sterilized using sterilizing agents, 70% ethanol, 0.5% sodium hypochlorite and sterile distilled water within specific period. Both explants, young immature leaves and male flower buds were cleaned under running tap water and rinsed with distilled water. Then, these explants were immersed in 70% ethanol for 30 seconds before being soaked in 0.5% sodium hypochlorite added with tween 20 for 8, 10 or 12 minutes. Later, both explants were rinsed with sterile distilled water for 3 -5 times.

2.3 Culture medium

The culture medium was prepared using MS basal salt supplemented with 3% (w/v) sucrose and different concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) and thidiazuron (TDZ) either alone or in combination. The concentrations of 2,4-D added was 0.0, 1.0, 3.0 and 5.0 mg/l while the concentration of TDZ was 0.0, 0.003, 0.005, 0.008 mg/l. Each treatment consist of 3 replicates and each plate contained 4 explants. The cultures were kept at 25 ± 2°C under dark condition.

2.4 Explant culture

The leaves were placed onto the sterilized A4 paper and cut into small square pieces of about 0.3 to 0.5 cm^2 prior to inoculation on media. Meanwhile for the flower buds, the mature flower buds were chosen and excised on the sterilized A4 paper. The sepals of the flower buds were removed to obtain the stamens. The stamens were also inoculated on the media for culture initiation. Observations on callus induction percentage and its morphology were recorded.

3. Results and Discussion

3.1 Explant Surface Sterilization

Plant tissue culture method must deal with contamination originating either from microorganisms on the surface, in the tissues of explants, or through flawed technical procedures in the laboratory. These are caused by several microbial contaminants, commonly bacteria and fungus (Cassells, 1991). In this experiment, the leaves and flower buds used as the explants were collected from plants that are exposed to surrounding, as they were planted at the nursery. The explants used were highly exposed to contaminants from other plants or the environment. Thus, proper surface sterilization protocols need to be done to reduce the possibilities of *in vitro* cultures contaminations.

Based on the observations made, the results obtained showed that leaf and flower explant treated with 70% ethanol for 30 seconds before being soaked in 0.5% sodium hypochlorite (added with tween 20) for 8 minutes has the highest rate of leaf explants survival with 82.5% and 95% respectively (table 1). Hence, this method is applied for the subsequent replications of the experiments. There were different source of microorganisms' contamination detected that causes death of leaves and flower explants in the *in vitro* culture.

Surface sterilization protocols usually comprise sterilizing agents that play important roles in controlling the growth of contaminants *in vitro*. The sterilizing agents include water, detergent solution, antibacterial agents, antifungal agents, sodium hypochlorite (NaOCl), calcium hypochlorite ($CaOCl_2$), ethanol, mercuric chloride ($HgCl_2$), and antibiotics (Smith, 2012). Guerrero-Abad et al. had optimised the sterilization methods of *in vitro* culture of sacha inchi leaves by treating with 70% ethanol for less than three seconds, followed by treatment with 0.5% sodium hypochlorite with ten minutes intervals and rinsed with sterile distilled water 3 to 4 times before inoculating in the media (Guerrero-Abad et al., 2010). Besides, the explants were cleaned under running tap water and distilled water after collection prior to surface sterilization process. These steps developed a contaminant free callus of sacha inchi.

In this study, both explants were treated with 70% ethanol for sterilization purpose. Generally, 70% ethanol is used as pre-treatment with other sterilizing agent and aid well in sterilization, but it is also known for its phototoxic effects and dehydration ability towards plant tissue (Mahmoud and Al-Ani, 2016). The effectiveness of ethanol is dependent on the exposure time, where overexposure can reduce the rate of success (Tewelde et al., 2020). For fragile and thin immature leaves and flower buds, both were optimized to be soaked in 70% ethanol for only 30 seconds as longer exposure was not effective in removing contaminants and might damage the explant tissues due to the phytotoxicity of the alcohol (Rodrigues et al., 2013). In addition, longer exposure to ethanol can lead to reverse osmosis where water flow out of the membrane causing the cells to shrink and lead to plasmolysis in explants (Sundram et al., 2012). Thickened plant tissues have lower chances to be damaged by alcohol, while unopened flower buds suggested for longer periods treatment with alcohol, since the tissue that will be cultured is within the structure that is being surface sterilized (Mahmoud and Al-Ani, 2016).

Next, both explants were treated with sodium hypochlorite (NaOCl) that are commonly used in surface sterilization for plant materials and proven to be effective to avoid bacterial contaminations (Sundram et al., 2012). This chemical is highly reactive against amino acid, nucleic acids, amides and amines as it has strong oxidizing property. Hence, it is highly significant in killing bacteria and fungi. Nevertheless, a suitable concentration, exposure time and other factors should be taken into consideration as it might give detrimental effects during surface sterilization process (Yildiz et al., 2012). Besides, Tween 20 that acts as surfactant, plays vital role in diminishing the infestation of possible surface contaminants and as wetting agent that disrupt the surface tension on the explants for good accessibility of sterilizing agents (Sundram et al., 2012).

In this study, ethanol parameter was kept constant while different exposure time of sodium hypochlorite were applied to explants. At this stage, Tween 20 was added to aid the sterilization process. It is found that exposure to 0.5% sodium hypochlorite for 8 minutes is the optimum value for sterilization of leaves explants. Meanwhile study stated that when same concentration of sodium hypochlorite used for ten minutes, the survival percentage of the explants was 100% (Guerrero-Abad et al., 2010). On the other hand, in this study, the male flower buds were also successfully sterilized with 0.5% of sodium hypochlorite that were exposed for 8 minutes. However, in contrast, the flower buds of *Manihot esculenta* treated with 10% sodium hypochlorite for 20 minutes has 100% survival rate (Buttibwa et al., 2020). Even though sacha inchi and cassava belong to the same Euphorbiaceae family, they are slightly different in size and the sepal thickness where sacha inchi flowers are smaller with thin sepals covering the anthers. Sodium hypochlorite effectiveness is directly proportional to its concentration and exposure time period, but with certain limitation or else it causes toxicity effects (Seran 2013).

Table 1: Percentage of survival of male explants under different exposure time to 0.5% sodium hypochlorite in combination with tween 20, 70% ethanol and sterile distilled water.

Explants	Exposure time to 0.5% Sodium Hypochlorite (min)	Percentage of survival (%)
Leaf	8	82.5
Leaf	10	2.5
Leaf	12	15.0
Flower	8	95.0
Flower	10	90.0
Flower	12	87.5

3.2 Explant Culture

After surface sterilization, the explants (leaves and male flowers) were cultured on medium supplemented with 2,4-D and TDZ to induce formation of callus. The result shows callus was not formed under the control treatment (T1 and K1) (Table 2 and table 3). Callus growth was also not observed under treatment T2, T3, T4, T5, T6 and T8 using leaf as explants (Table 2). As for leaf explants, only treatment T7 supplemented with 1.0mg/L 2,4-D in combination with 0.005 mg/L TDZ and 3% sucrose shows callus growth as in Figure 1 (Table 2). The explant in this treatment produced friable creamy white callus (Figure 1) growing at the edge of the leaf cuttings, 4 weeks after culturing. As for male flower explants, friable creamy white callus were observed under treatment K6, K7 and K8 as shown in figure 3b, figure 3c and figure 3d respectively while treatment K5 produced friable pure white callus as shown in figure 3a (Table 3).

Treatment K7 induced the biggest callus with the size 3.0cm^2. Meanwhile, male flower explants in treatments without 2,4-D (K1, K2 and K3) did not produce any callus except for treatment K4 supplemented with 0.008 mg/L TDZ produced friable brownish callus (Figure 2).

Plant growth regulator 2,4-D is a synthetic auxin, that offers better stability than Indole-3-acetic acid (IAA), the naturally occurring auxin. It is required by plant cell for division and root initiation. In plant *in vitro* culture, 2,4-D is widely used for callus induction as it revert the explant cells to a dedifferentiated state and begins to divide (Dalila et al., 2013). Whereas cytokinin, particularly TDZ enhance the growth and differentiation of cultivated explants. TDZ exhibits the unique property mimicking both the auxin and cytokinin effects although it is structurally different from either auxin or purine-based cytokinins (Murthy et al., 1998). This hormone proven to induce organogenesis directly or indirectly, thus promoting regeneration in Euphorbiaceae plants (Restrepo-Osorio et al., 2020). Theoretically, equal ratio of auxins and cytokinins facilitate callus induction; nevertheless, this differs greatly in practice due to differences in the endogenous levels of phytohormones in individual plants (Kumlay and Ercisli, 2015). When 2,4-D at 2.0 mg/L combined with TDZ ranging from 0.0 to 0. 1 mg/L, callus were formed where leaves of sacha inchi under treatment of 2.0 mg/L 2.4-D and 0.005 mg/L TDZ shows the best response (Guerrero-Abad et al., 2010). Meanwhile, the culture of *Manihot esculanta* anther male flowers treated with 2,4-D ranging from 2.0 to 5.0 mg/L developed callus under 24 hours dark condition (Buttibwa et al., 2020).

Table 2: Effect of 2,4-D and TDZ on callus induction using leaf explants. fc: friable callus, cw: creamy white callus

Treatment	2,4-D (mg/l)	TDZ (mg/l)	Morphology of callus	Size of callus (cm^2)
T1	0.0	0.000	Callus not formed	-
T2		0.003	Callus not formed	-
T3		0.005	Callus not formed	-
T4		0.008	Callus not formed	-
T5	1.0	0.000	Callus not formed	-
T6		0.003	Callus not formed	-
T7		0.005	fc; cw (Figure 1)	1.5
T8		0.008	Callus not formed	-

Table 3: Effect of 2,4-D and TDZ on callus induction of male flower explants. fc: friable callus, cc: compact callus, cw: creamy white, bc: brownish callus, pw: pure white callus.

Treatment	2,4-D (mg/l)	TDZ (mg/l)	Morphology of callus	Size of callus (cm^2)
K1	0.0	0.000	Callus not formed	-
K2		0.003	Callus not formed	-
K3		0.005	Callus not formed	-
K4		0.008	fc; bc (Figure 2)	1.5
K5	1.0	0.000	fc; pw (Figure 3(a))	2.5
K6		0.003	fc; cw (Figure 3 (b))	2.5
K7		0.005	fc; cw (Figure 3(c))	3.0
K8		0.008	cc; cw (Figure 3 (d))	0.7

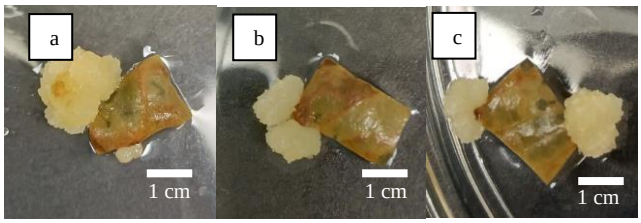

Figure 1: Friable and creamy white callus formed on 4 weeks sacha inchi *in vitro* leaf cultures on MS medium supplemented with 3% sucrose, 1.0 mg/L of 2,4-D and 0.005 mg/L TDZ.

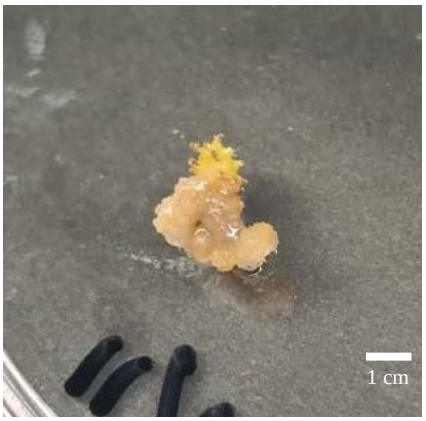

Figure 2: Friable and brownish callus formed on sacha inchi *in vitro* male flowers cultures after 4 weeks on MS medium supplemented with 3% sucrose and 0.008 mg/L TDZ.

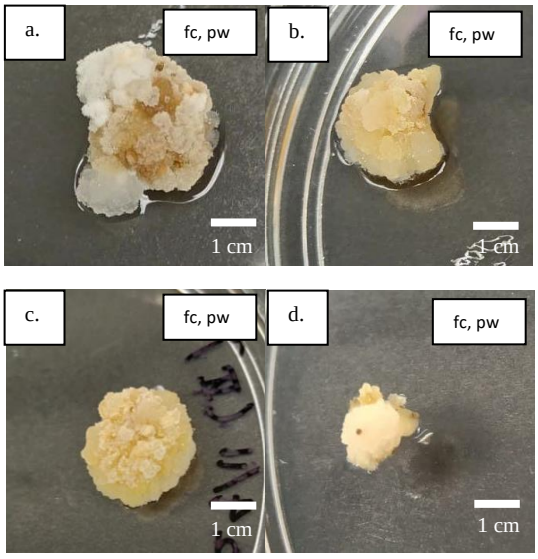

Figure 3: Callus formed on sacha inchi *in vitro* male flowers cultures after 4 weeks on MS medium supplemented with 3% sucrose and 1.0mg/L 2,4-D in combination with 0.000mg/L, 0.003 mg/L, 0.005 mg/L and 0.008 mg/L TDZ, respectively. (a) Friable and pure white callus. (b) Friable and creamy white callus. (c) Friable and creamy white callus. (d) Friable and creamy white callus.

4. Conclusion

In conclusion, explant treated with 70% ethanol for 30 seconds before being soaked in 0.5% sodium hypochlorite for 8 minutes has the highest rate of leaf and flower explants survival with 82.5% and 95% respectively. This study also showed that callus could be successfully formed on MS medium supplemented with 3% sucrose and different concentrations of plant growth regulators 2,4-D and TDZ. For both, leaf and male flower explants, MS medium supplemented with 3% (w/v) sucrose in combination with 1.0 mg/L 2,4-D and 0.005 mg/L TDZ recorded the best response in term of plant growth regulators. This treatment developed friable callus. Besides, when male flower was used as explant, increase of TDZ to 0.008 mg/L was seen to cause reduction of the callus size and multiplication rate. Hence, this study proved callus induction using leaf and male flowers are possible using different plant growth regulators,

producing different effects on the morphology. Further study can be done to obtain suspension cell culture using callus culture of sacha inchi for production of the bioactive compounds.

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