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Optimization of Sterilization and Regeneration Protocol in Micropropagation of *Agave angustifolia*

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ABSTRACT

***Agave angustifolia* is one of the economically important plant, which is not commonly used in Sri Lanka. Agave plants can be used as live fences, hence, there is a large demand for uniform planting materials. Micropropagation is the only technique to get uniform and cleaned planting materials in mass scale. Optimized tissue culture protocol for micropropagation of *Agave angustifolia* is not available. As the first step, establishment of an efficient surface sterilization protocol for the field collected material is a primary requirement. Effect of 2, 4-Dichlorophenoxy acetic acid (2, 4-D; 0, 0.1, 0.5 mg/L) and 6-Benzylaminopurine (BAP; 0, 1, 2, 5, 10 mg/L) alone or combinations were tested for regeneration or callus induction. Mercuric chloride ($HgCl_2$) is very efficient in disinfection, however, it is hazardous to the environment and the human. Therefore, identification of an alternative protocol is highly important. This study was carried out to determine suitable contamination control protocol and furthermore, a suitable multiplication medium. Results revealed that the surface sterilization is efficient either with 0.1% $HgCl_2$ for 10 min or dip in 70% ethanol followed by 20% sodium hypochlorite for 20 min. However, considering the health hazardousness, axillary shoot development and tissue viability, the latter protocol is highly efficient. None of the growth regulators or the tested concentrations were not effective in enhancing the regeneration or callus induction.**

KEYWORDS: *Agave angustifolia*, Live fence, Micropropagation, Multiplication, Sterilization

INTRODUCTION

Genus *Agave* is the most respective of the family Agavaceae that includes about 200 species, 75% of which are distributed in Mexico (Garcia-Mendoza, 2000; Garcia-Mendoza, 2004). *Agave angustifolia* is a famous agave species which is originated in Mexico and most widely distributed in the world. It is commercially grown for Mezcal (Bacanora) production (Palomino *et al.*, 2007) an aroma content distillate (Gentry, 1982; Gutierrez *et al.*, 2007). *Agave angustifolia* contains steroids for pharmaceutical products and high content of sucrose, glucose and inulin (Robert *et al.*, 2004). It is used as an animal feeds, fiber production, a food, a medicine and as an ornamental plant. In Sri Lanka it is used only as an ornamental plant.

This plant has a long life cycle that takes about 8-20 years (Cervantes *et al.*, 2007; Robert *et al.*, 2004). A thick spine available at the leaf tip makes the plant to be used as live fences. Environment Forest Conservation Unit of Mahaweli Authority Sri Lanka tries to apply this plant as the elephant barrier which is an alternative to the electric fence to control the movement of the elephants out of the jungles. Elephant and human attack is a serious problem in Sri Lanka. Electric fence that are used for this purpose is not functioning properly and it is not a good solution for this problem. It is harmful for both animal and human. It has been noticed that elephants don't like to get closer to the *A. angustifolia* plants, therefore, it can be used an elephant barrier or as a live fence for elephants. Agave plants are required in mass scale for this purpose if they have to be used when making the fence. However, finding sufficient number of agave plants is very difficult.

Agave plant propagate through both sexual and asexual reproduction (Robert *et al.*, 2004). It produces seeds, however, sexual reproduction mechanism is insufficient for commercial cultivation (Robert *et al.*, 2004). Sexual reproduction mainly depends on environmental condition, especially temperature and humidity. However, sexual reproduction is occurring rarely, and the plants may not similar to the mother plants due to the changes occur in the genetic composition. Therefore getting clones of *A. angustifolia* through vegetative reproduction is very important. The plant usually propagated by suckers and bulbils (Gentry, 1982; Nobel, 1998). Natural vegetative propagation of agave is insufficient, to supply the demand, therefore, mass propagation through tissue culture is the only solution. Thousands of healthy clonal plants with minimum unwanted genetic variability can be propagated in few months from a single mother plant through tissue culture.

Thus, the present study was undertaken to develop a protocol for mass production of

agave. The first requirement to initiate a tissue culture protocol is establishment of an efficient disinfection method for the plant materials collected from the fields to minimize the wastage by contamination and in the study emphasis was given to establish a sterilization protocol.

MATERIALS AND METHODS

Location

The experiment was carried out in the tissue culture laboratory at the Department of Environment and Forest Conservation, Mahaweli Authority of Sri Lanka, Mawathura, from January to May 2016.

Sample Collection

Freshly uprooted healthy mature or semi mature mother plants consisting about 50 leaves were collected from Mawathura and Doluwa area.

Sample Preparation and Sterilization

The leaves were removed carefully and the stems were cut into several pieces. Stem pieces were washed with teepol and kept under running tap water for 20 min for pre surface sterilization. Axillary buds with 1×1 cm base were excised from the stem cuttings under aseptic condition. Four surface sterilization methods (Table 1) were tested to identify the best method for controlling contamination with least affected for plant regeneration.

Table 1. Tested protocols for controlling contamination of cultured agave axillary buds

Sterilization Protocol	Procedure
1	10% NaOCl (20 min); 0.07% carbendazim fungicide (20 min); 0.1% $HgCl_2$ (10 min)
2	10% NaOCl (20 min
3	70% EtOH (1 min); 10% NaOCl (20 min)
4	70% EtOH (1 min); 20% NaOCl (20 min)

NaOCl- Sodium hypochlorite, $HgCl_2$- Mercuric chloride, EtOH- Ethyl alcohol, min-minute

After application of each disinfectant the explants were washed three times with sterilized distilled water.

Culture Initiation

Axillary buds were cultured in the culture tubes containing MS basal medium (Murashige and Skoog, 1962) supplemented with 0.5 mg/L GA_3, 5 mg/L BAP and 5mg/L sucrose. Medium pH was adjusted to 5.6, solidified with 0.6% (w/v) agar and dispensed into the culture tubes. After removing all unnecessary stem tissues, the axillary buds were cultured in to each culture tube. Ten axillary buds were used for each treatment of sterilization protocol. Culture tubes were maintained at 25 °C and photoperiod of 12/12. Experiment was repeated three times. Observations were made weekly for one month period. Contamination, shoot development, dead buds and nonresponsive buds were recorded.

Effect of Growth Regulators

Different concentrations of growth regulators 2, 4-D (0, 0.1 and 0.5 mg/L) and BAP (0, 1, 2, 5, 10 mg/L) were tested for their response for shoot multiplication or callus induction. Cultures were maintained in the dark. Experiment was repeated three times. Data was recorded weekly for two months.

Data Analysis

Data were statistically analyzed using maximum likelihood analysis using Statistical Analysis System (SAS) version 9.2.

RESULTS AND DISCUSSION

After excision from the mother plant, the explants need to be surface disinfected. The fragments of plant tissue should be left as large as possible for disinfection, to minimize damage to tissues by the disinfectant (Gratton and Fay, 1999), however, unnecessary parts should be removed after sterilization before culturing.

Result revealed that the contamination rate of protocol 1, 3 and 4 were not significantly different (Table 2) indicating that they are equally effective for sterilization. The highest contamination rate (50%) was recorded in protocol 2 with only 10% NaOCl. It was significantly different from protocol 1 (4.54%; $P<0.05$) and protocol 4 (13.63%; $P<0.05$) indicating that low concentration of NaOCl (10%) is not sufficient for disinfecting the extracted plant material. Protocol 1 is consisted of $HgCl_2$ that is a heavy toxic compound and health hazardous. Sodium hypochlorite in high concentration together with 70% ethanol (protocol 4) was effective equally with the protocol consists of $HgCl_2$ in controlling the contamination. Considering the safety issues, protocol 4 can be selected as the best protocol for disinfection of agave axillary buds.

Furthermore, the highest regeneration rate (43.9%) was recorded in protocol 4, over protocol 1 in which the lowest regeneration rate (2.43%) was recorded, revealing the negative effect of $HgCl_2$ for regeneration. The highest death rate of the tissues was observed in protocol 1 (50%) and minimum in protocol 4

Table 2. Response of the cultivated axillary buds after each sterilization protocol

Sterilization protocol	Contamination rate (%)	Regeneration rate (%)	Death rate (%)	Uninitiated rate (%)
Protocol 1	4.54[a]	2.43[a]	50.00[a]	65.21[a]
Protocol 2	50.00[b]	24.30[b]	26.92[b]	8.69[b]
Protocol 3	31.81[ab]	29.26[b]	15.30[b]	30.43[ab]
Protocol 4	13.63[a]	43.90[b]	11.53[b]	17.24[ab]

Means in a column with the same letters are not significantly different at the 0.05 level; n=30

(11.53%). Cultured buds remained without any response in protocol 1 (65.2%) over the others.

Reason for low regeneration efficiency and high browning with protocol 1 could be due to the heavy tissue damage. However, Nikam *et al.* (2003) reported that in *Agave angustifolia* showed a high germination rate of 75.7%.

Highest axillary buds which were not contaminated, dead and uninitiated rate recorded in protocol 1 indicating its negative effect on the purpose whereas protocol 4 performed better in all the aspects as well as regeneration efficiency.

Therefore, suitable contamination control protocol for *Agave angustifolia* tissue culture and multiplication is the protocol 4. Even though $HgCl_2$ is normally used for contamination control, it is hazarded to the environment. Therefore finding more suitable contamination control method which exempted $HgCl_2$ is important.

Effect of Growth Regulators for Shoot Multiplication or Callus Induction

Effect of two growth regulators, BAP and 2,4-D were tested for direct shoot multiplication or regeneration through somatic embryogenesis. Results revealed that there is no any significant effect of adding any concentration of BAP in to the culture medium for shoot multiplication.

Results also indicated that incorporation of 2,4-D was negatively affected for shoot development showing the highest bud formation (60%; $P < 0.05$) in the medium devoid any growth regulators (Table 3). Santacruz *et al.* (1999) also reported that 2,4-D drastically inhibited shoot proliferation in *Agave parrasana.* Among the tested concentrations of 2,4-D, no one was effective even in callus induction. However, with the increased 2,4-D concentration tissue deformation was observed.

Table 3. Effect of 2, 4-D for bud formation

2,4-D level (mg/L)	Bud formation rate (%)
0.0	60[a]
0.1	20[b]
0.5	20[b]

Means in a column with the same letters are not significantly different at the 0.05 level; n=48; 2, 4-D - 2, 4-Dichlorophenoxy acetic acid

CONCLUSIONS

According to the results, protocol 4 (70% EtOH for 1 min; 20% NaOCl for 20 min) is the best for controlling the contamination and inducing regeneration in *Agave angustifolia.* Mercuric chloride was effective in controlling the contamination, however, negatively affected for tissue viability and regeneration. Tested BAP and 2,4-D concentrations were not effective in enhancing the regeneration or callogenesis efficiency.

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