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Standardisation of surface sterilization procedures for micropropagation in *Heliconia stricta* var. *Iris bannochie*

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ABSTRACT

Heliconia also popularly known as lobster-claw wild plantain or false bird of paradise. *Heliconias* are plants of commercial interest as garden or cut flowers. *Heliconia* flowers are actually highly modified leaves and bracts. *Heliconia* come in two main orientations erect and pendent. The flowering stems are mostly pendulous. The micro propagation of *Heliconia* to produce healthy propagates. The explants were then washed under running tap water to remove surface contaminants. Various treatments were given to sterilize the explants of *Heliconia* because of high degree of contaminant load. To obtain contamination free culture, the following five treatments were tried out. The experiments were conducted using the rhizome explants of *Heliconia stricta var. Iris bannochie;* to study the effectiveness of sterilization procedures using various sterilants given in varying concentrations and exposure durations. In the present study three sterilizing agents viz ethanol, NaOCl and HgCl2 were used, giving varying exposure time and concentrations. The survival rate was very low due to long exposure time of the sterilizing agents. It leads to gradual darkening of the explants until death, may be due to oxidation.

Key words: *Heliconia stricta*, Ornamental market, micropropagation.

INTRODUCTION

Heliconias are one of the main families of tropical plants related to bananas, cannas and gingers. *Heliconias* were first discovered, they were first classified as part of the banana family Musaceae because of the similarities in leaves and growing patterns. *Heliconia* is now the only genus under Heliconiaceae.*Heliconia* also popularly known as lobster-claw wild plantain or false bird of paradise. *Heliconias* are plants of commercial interest as garden or cut flowers. Depending on the species, their height varies between 0.5 to 8.0 m. Among the tropical flowers. *Heliconias* out standing for its diversity in form, color, size and particularly its durability. These characteristics give the Heliconiaceae family great potential in the ornamentals market. The typical diversity of *Heliconias* is something that drives floriculture as a whole.

Heliconia flowers are actually highly modified leaves and bracts. *Heliconia* come in two main orientations erect and pendent. The flowering stems are mostly pendulous. *Heliconia* flowers are produced on long, erect or drooping panicles, and consist of brightly colored waxy bracts, with small true flowers inside the bracts. To survive and grow properly *invitro* plant cultures need to be largely free of fungi and bacterial infections. Contamination can cause large losses during micropropagation and their control usually the most frequent and difficult problem encountered by micropropagation laboratories. Constantine (1986) reported that plant tissue cultures have been said to be liable to three kinds of contamination.

1. The sudden and severe kind (acute contamination) which occurs during the establishment of a culture. This is nearly caused by ineffective surface sterilization.
2. Contamination that occurs after establishment caused by microorganisms

that were concealed within the explants. (Endogenous contamination)

3. Micro organisms that have been introduced during sub culturing.

One of the methods to control the contamination rate is by the use of antibiotic. Hence it is highly necessary to reduce and control the contamination rate in commercial micro propagation to increase the quality of culture.

Micropropagation of *Heliconia*

Heliconia cultivation is presenting now a days, significant expansion in small and medium forms due to the beauty and high value of flowers in the ornamental market. This market which is stimulated by novelties and depends on the natural variety and different techniques that are developed in order to generate new products. The cultivation of plant tissues represents an important source of variety, producing different types of Somoclonal variants. The reduced propagates often makes the cost of production high in Brazil (Lamas, 2001).

Quite a few research centers have dedicated efforts to study the aspects related to the micro propagation of *Heliconia* to produce healthy propagates. Biotechnological methods might contribute to solve some constraints for Heliconia cultivation such as the long period required for seed germination (from three months to one year) and the underground rhizome multiplication that takes a long time and cannot be properly followed up (Atehortua, 1997).

Mineral elements

Macronutrients

Elements which are required in the concentration greater than 0.5mM known as macronutrients. These include six major elements Nitrogen (N), Potassium (K), Phosphorus (P), Calcium (Ca), Magnesium (Mg) and Sulphur (S).

Micronutrients

The micronutrients consist of six elements at below 0.5 mM in concentration. The elements are Iron (Fe), Manganese (Mn), Zinc (Zn), Boron (B), Copper (Cu) and Molybdenum (Mo). These are all required in very trace quantities for the growth of tissues.

Organic compounds

Organic compounds are also added to plant culture medium. Some of these compounds such as sugar are needed for growth, while others such as vitamins, amino acids etc may not be essential but may enhance growth.

Sugar

Most plant tissues are not highly autotrophic that is capable of fixing carbon through photosynthesis due to limitations of carbon dioxide availability among other factors. Therefore sugar added to the medium serve as an energy source. Sucrose is the most common sugar added. It is the sugar form most commonly transported in plants. It is broken down into glucose and fructose during metabolism. The concentration of sugars in nutrient media generally ranges from 20 to 40 g/l.

Vitamins

Thiamine (vitamin B1) is required for carbohydrate metabolism and the biosynthesis of some amino acids has been shown to be essential for most plant cultures. Nicotinic acid (niacin) and Pyridoxine (B6) are also commonly added to Murashige and Skoog medium. Other vitamins such as Ascorbic acid (vitamin C), Folic acid, Biotin and Vitamin E (tocopherol) are sometimes added to media formulations. Vitamin concentration is generally low.

Myo inositol

Myo inositol is a sugar alcohol, added to most plant culture media that can significantly improve *in vitro* response.

Amino acids

Although nitrogen source are present in the inorganic salts, various amino acids and amides are used in plant tissue culture media. Amino acids like Glycine which acts as an additional nitrogen source to the plant cells are used in small amounts.

Plant growth regulators (phytohormones)

Plant growth regulators are small organic molecule that elicits physiological response at very low concentrations. Phytohormones regulate the growth of plants, help in differentiation of cells etc. The important plant growth regulators which are used in tissue culture media include Auxins and Cytokinins.

Auxins

Auxins stimulate cell elongation, differentiation of vascular tissues, initiation of roots etc. Mainly used auxins are IAA - Indole Acetic Acid, IBA - Indole 3 Butyric Acid, NAA - Naphthalene Acetic Acid.

Cytokinins

Cytokinins help in the elongation of root and stem and it promote cell division and organ formation. Zeatin is the naturally occurring cytokinin. The cytokinins which are used as growth regulators in tissue culture media are BAP and kinetin.

Solidifying agent

Agar

Solidifying agents are used to create semisolid or solid media. Agar is the most commonly used gelling agent. It is a polysaccharide mixture from extract of several species of red algae. Certain other gelling agents used are agarose, gelrite, phytagel etc.

pH

pH of the medium is usually adjusted between 5.5 to 6.0 before sterilization. This adjusted by using 1N HCl or 1N NaOH. In general pH higher than 6 usually gives a fairly hard medium and a pH below 5 does allow satisfactory gelling.

Media preparation

Cultures were established in MS media supplemented with coconut water. Separate stock solutions of macronutrients, micronutrients, vitamins, amino acids, calcium and iron were prepared in double distilled water. The stock solution prepared for media preparation is shown in Table 1.

Sterile techniques

Glass wares used in the present study were rinsed with distilled water and autoclaved before use. The bottles containing the culture media, the glass wares and liquids were autoclaved under moist heat and pressure of 15lbs and a temperature at 121°C for 15 to 20 minutes in a vertical autoclave. The inoculation room was provided with LFC with UV source and HEPA filter to ensure aseptic working condition. The germicidal lamps in the LFC and inoculation room were switched on for 20 minutes to reduce the microbial load. In addition to this the working area of LFC was wiped with 70% ethanol. The scalpels and forceps used for inoculation were autoclaved prior after wrapping in Aluminium foil. The forceps and scalpels were flamed at periodical intervals during inoculation after dipping in 99% ethanol. The inoculated culture vessels are maintained in the culture rooms provided with culture racks containing fluorescent lamp fitting. The required temperature of 24°C was constantly maintained with the help of air conditioners and the relative humidity is periodically observed and checked using the hygrometer.

***Invitro* procedure**

The mother plants were given a foliar spray of 500 ppm streptocycline sulphate before one week of explants collection. The material used as explants in the present study consisted of *Heliconia stricta var* Iris *bannochie* rhizomes. The rhizomes were collected from the disease free shoots of Heliconia; Older leaves and extraneous tissues were trimmed to a convenient size of 2 to 3 cm and transferred to the laboratory.

The explants were then washed under running tap water to remove surface contaminants. Again the explants were washed with sterile water containing two drops of emulsifier (Tween 20) followed by three times rinse with sterile water. Remaining surface sterilization procedures were carried out in LFC. Various treatments were given to sterilize the explants of Heliconia because of high degree of contaminant load. To obtain contamination free culture, the following five treatments were tried out.

Treatment 1

The explants were dipped in 70% ethanol for two seconds and rinsed three times with sterile deionized water. It was followed by disinfection with 5% NaOCl for ten minutes and rinsed with sterile water three times. Thereafter the explants were soaked in 0.1% HgCl2 for three minutes and rinsed three times with sterile water. Sides of the explants were trimmed to remove dead tissues. Trimmed explants were dipped in CA water for three seconds. The surface sterilized explants were then inoculated aseptically into the culture media using flame sterilized forceps.

Treatment 2

The explants were soaked in 70% ethanol for 4 seconds and rinsed three times with sterile water. After that the explants were kept immersed in 10% NaOCl for ten minutes and rinsed thoroughly with sterile water three times. Thereafter the explants were treated with 0.1% HgCl2 for 6 minutes and rinsed three times with sterile water. Sides of the explants were cut to remove dead tissues and the size was reduced to 2 cm and dipped in CA water for 3 seconds to reduce browning. Surface sterilized explants were inoculated to the media containing 2 drops of streptocycline using a sterile forceps.

Treatment 3

The explants were dipped in 70% ethanol for six seconds. Then rinsed three times in sterile water followed by disinfection by immersion in 20% NaOCl for 10 minutes. Explants were thereafter rinsed three times with sterile water. After that the rhizomes were kept immersed in 0.1% HgCl2 for nine minutes and rinsed three times with sterile water. After disinfection the explants were trimmed to 2cm size to remove the dead tissues. Trimmed explants were dipped in CA water for three seconds; inoculated in the media containing two drops of vancomycin using a flame sterilized forceps.

Treatment 4

The explants were soaked in 70% ethanol for eight seconds and rinsed three times with sterile deionised water. Thereafter kept immersed in a solution of 30% NaOCl for ten minutes followed by washing three times with sterile water. After that the explants were soaked in o.1% HgCl2 for twelve minutes and rinsed thoroughly three times with sterile water. Sides of the explants which were in contact with the HgCl2 were cut using a sterile scalpel. Trimmed explants were dipped in CA water for three seconds and inoculated in the media containing two drops of tetracycline using a sterile forceps.

Treatment 5

In T5 after the initial wash with tween 20, the explants were immersed in a 2% solution of bavastin for fifteen minutes, rinsed with sterile water and then transfered to LFC. The explants were then dipped in 70% ethanol for ten seconds and rinsed with sterile water three times. The rhizomes were then kept immersed in a solution of 40% NaOCl for ten minutes and washed with sterile water three times. It was followed by disinfection with 0.1% HgCl2 solution for fifteen minutes, rinsed three times with sterile water. Sides of the explants were trimmed to remove extraneous tissues, dipped in CA water for three seconds and inoculated in the media devoid of antibiotics using a sterile forceps.

RESULTS AND DISCUSSION

The experiments were conducted using the rhizome explants of *Heliconia stricta var*. Iris *bannochie;* to study the effectiveness of sterilization procedures using various sterilants given in varying concentrations and exposure durations. Surfaces of plants carry a wide range of microbial contaminants. To reduce the microbial load the explants tissues must be thoroughly surface sterilized before inoculating it on the nutrient medium. Explants treated with Tween 20, a wetting agent improved the disinfection by acting as a surfactant thereby removing the surface contaminants like soil and dust.

NaOCl diluted to 10 to 20% of the original concentration and immersion of plant material for 10 to 20 minutes was found to be a better way for disinfection. HgCl2 was found to be a very effective sterilizing agent at 0.1% concentration because of its high penetrating power. In order to control the phenol exudation from the trimmed tissues the explants were given anti browning treatment. CA water was found to be better for reducing browning of explants.

In the present study three sterilizing agents viz ethanol, NaOCl and HgCl2 were used, giving varying exposure time and concentrations. In T1 when 70% ethanol dip for two seconds, 5% NaOCl for ten minutes and 0.1% HgCl2 for three minutes were given, the explants showed high degree of bacterial contamination and low mortality rate. Explants were able to tolerate the sterilization procedure well due to low concentration of sterilants. But due to the high load of contaminants cultures started dying fourteen days after inoculation.

Table 1: Composition of ms media preparation

S. No.	Stock	Required per liter mg/l	Required for stock	Final stock volume	concentration	Volume required forworking solution (1000 ml)
A. Major Nutrients						
1.	Ammonium nitrate ($NH_4 NO_3$)	1650	16.5g	250ml	40x	25ml
2.	Potassium dihydrogen orthophosphate($KH_2 PO_4$)	170	1.7g			
3.	Potassium nitrate(KNO_3)	1900	19g			
4.	Magnesium Sulphate ($MgSO_4 7H_2O$	370	3.7g			
B	Calcium Chloride $CaCl_2 2H_2o$	440	8.8g	100ml	200x	5ml
C. Minor Nutrients						
1.	Manganese Sulphate ($MNSO_4 7H_2O$)	22.3	557.5 mg	250ml	100x	10ml
2.	Zinc Sulphate ($ZnSO_4 7H_2O$)	8.6	215 mg			
3.	Boric Acid ($H3BO_3$)	6.3	157.5mg			
D. Micro Nutrients						
1.	Sodium molybdate ($Na_3MOO_4 2H_2O$)	0.25	12.5mg	100ml	500x	2ml
2.	Cobaltous Chloride ($CoCl_{26}H_2O$)	0.025	1.25mg			
3.	Cupric Sulphate ($CuSO_4 5H_2O$)	0.025	1.25mg			
E.	Potassium iodide (KI)	0.83	41.5mg	100ml	10x	100ml
F. Iron						
1.	Ferrous Sulphate ($FeSO_4 5H_2O$)	27.8	27.8	100ml	10x	100ml
2.	Disodium EDTA (Na2EDTA)	37.3	37.3			
E. Vitamins and amino Acid						
1.	Nicotinic Acid	0.5	25mg	100ml	500x	2ml
2.	Thiamine HCl	1	10mg	10ml	10x	1ml
3.	Pyridoxine HCl	0.5	25mg	100ml	500x	2ml
4.	Glycine	2	20mg	100ml	500x	10ml
	Meso inositol	100mg				
	BA	2mg	10mg	10ml	500x	2ml
	NAA	0.05mg	10ml	10ml	20,000x	0.05ml
	Sucrose	30g				
	Coconut Water	100ml				
F.	Agar	7g				
G.	pH	5.7 – 5.8				

In T2 when 70% ethanol dip for 4 seconds, 10% NaOCl for ten minutes, 0.1% $HgCl_2$ for six minutes and two drops of streptocycline were given, decrease in contamination was observed. Out of the ten cultures initiated, four showed contamination free cultures during the initiation phase. But during sub culture when the sides of the explants were trimmed further to remove the dead tissues they also started bacterial contamination. This may be due to the absence of the antibiotic streptocycline in the sub cultured medium which was preventing the endophytic bacteria present in Heliconia explants.

In T3 when 70% ethanol for six seconds, 20% NaOCl for ten minutes, 0.1% $HgCl_2$ for nine minutes and two drops of vancomycin were used contamination was very low and the survival rate was also 50%. Only 30% plants showed mortality due to sterilization. The survived contamination free cultures exhibited throwing symptoms. Antibiotic vancomycin was effective in controlling the endophytic bacteria present in *Heliconia.*

Table 2: Treatment 1

Product	Concentration	Exposure
Ethanol	70%	2Sec
NaOCl	5%	10min
$HgCl_2$	0.1%	3min
Antibiotic	Nil	

Table 3: Treatment 2

Product	Concentration	Exposure
Ethanol	70%	4Sec
NaOCl	10%	10min
$HgCl_2$	0.1%	6min
Antibiotic	Streptocyclin	

Table 4: Treatment 3

Product	Concentration	Exposure
Ethanol	70%	6Sec
NaOCl	20%	10min
$HgCl_2$	0.1%	9 min
Antibiotic	Vancomycin	

Table 5: Treatment 4

Product	Concentration	Exposure
Ethanol	70%	8Sec
NaOCl	30%	10min
$HgCl_2$	0.1%	12min
Antibiotic	Tetracycline	

Table 6: Treatment 5

Product	Concentration	Exposure
Ethanol	70%	10Sec
NaOCl	40%	10min
$HgCl_2$	0.1%	15min
Bavastin	2%	15min

Table 7: Effect of various treatments

Treatment	Survival	Contamination	Mortality
T1	2/10	6/10	2/10
T2	4/10	4/10	2/10
T3	5/10	2/10	3/10
T4	2/10	1/10	7/10
T5	0/10	1/10	9/10

In T4, 70% ethanol for eight seconds, 30% NaOCl for ten minutes, 0.1% HgCl2 for twelve minutes and two drops of tetracycline were used. Only 10% cultures were contaminated but due to the severity of sterilization, 70% of the cultures showed dying symptoms and turned black. In T5 explants were dipped in 2% solution of bavastin before the initiation of sterilization procedure. Here the exposure time and concentration of the sterilant was the highest. 90% of the cultures showed mortality due to high concentration of sterilants but only 10% cultures exhibited contamination.

From the observation taken; it can be concluded that the sterilization procedure involving 70% ethanol dip for six seconds combined with 20% NaOCl for twenty minutes and 0.1% HgCl2 for nine minutes and the addition of antibiotic vancomycin in the initiation medium can produce contamination free culture in Heliconia.

Summary

The five treatments tried out, T3 was found to be the most effective one. Contamination rate was very low and the survival rate was high in T3. It may be due to the balance between the concentration and exposure time of the sterilants.In T1 and T2 the plants were able to tolerate the sterilization procedures, but showed high degree of contamination. It may be due to the low concentration of the sterilants.In T4 and T5; the survival rate was very low due to long exposure time of the sterilizing agents. It leads to gradual darkening of the explants until death, may be due to oxidation.

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