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RESEARCH ARTICLE

CSUP technique: a low cost sterilization method using sodium hypochlorite to replace the use of expensive equipment in micropropagation

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Abstract: The ability of *in vitro* micropropagtion to produce high quality planting material in large quantities is hindered by their high cost of production. Hence the price of planting material is high, making them unaffordable to growers. However, a larger portion of the capital and running cost of micropropagation can be reduced using CSUP (pronounced as seesap) technique, where sodium hypochlorite is used to sterilize glassware and culture media followed by culturing without using a laminar flow cabinet. This study was undertaken with the objective of evaluating the feasibility of the CSUP technique to replace the sterilization carried out using autoclaves and laminar flow cabinets.

Sodium hypochlorite in four concentrations 5, 10, 15 and 20% (v/v) were used to rinse glassware and accessories prior to pouring the appropriate culture medium for anthurium cv 'Tropical Red' *in vitro* shoot multiplication. The culturing of axenic shoots was carried out inside a glass-cage and cultures were placed in the growth room. Autoclaved culture medium and a laminar flow cabinet were used in the case of the control cultures. Results revealed that the percentage of contamination-free cultures, dry weight increase and the shoot multiplication were not significantly different between the respective treatments and the controls, as well as among treatments of different sodium hypochlorite concentrations. Therefore, the CSUP technique using 5% (v/v) sodium hypochlorite solution can be adopted to replace the use of an autoclave and the laminar flow cabinet in the major micropropagation activity of *in vitro* multiplication.

Keywords: CSUP, low cost micropropagation, sodium hypochlorite.

INTRODUCTION

High quality planting material is the key to achieve success in commercial agriculture. Superior planting material gives high yield and high quality produce with lengthy post harvest life. Thus production of custom made planting material paves way to high profit. The way to produce such planting material is through the use of *in vitro* micropropagation technique. The ability to produce commercially viable quantities and the ability to programme the production are the most advantageous aspects of this technique when compared with the conventional techniques, which produce clones (Debergh, 1987). Being a sophisticated method, *in vitro* micropropagation needs high capital and running cost, which makes the production cost of plants higher than the conventionally produced planting material. This situation has become a barrier for industry to use superior quality planting material.

There have to be proper resources and facilities for *in vitro* micropropagation of planting material. They include a well equipped laboratory, a protected structure and trained personnel. The laboratory requires specific equipment, supplies and services. However, small scale growers may not have such facilities as the equipment cost is very high. Therefore, many low cost options, which can be adopted by growers in developing countries, have been investigated (IAEA, 2004; Sood & Chauhan, 2009). There are no reports on alternatives to replace

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the use of expensive equipment such as autoclaves and laminar flow cabinets in *in vitro* micropropagation. However, the CSUPW method, an alternative technique, which does not require the use of an autoclave and a laminar flow cabinet was developed (Peiris & Peiris, 1998) and used successfully for commercially important crops such as tomato, cucumber, bell pepper, chinese cabbage, *Momordica*, strawberry, pineapple, gerbera, carnation, Madonna lily, *Gypsophilla, Hosta*, *Dracaena, Syngonium*, orchid and anthurium (Peiris *et al.*,1999). However, when using this technique the composition of the solution used had to be changed due to unavailability of the chemicals used in CSUPW technique. Preliminary attempts, to find suitable chemical/s, which are available locally revealed that sterilizing glassware with a solution of sodium hypochlorite (known as household bleach) was successful and that culturing to produce axenic cultures can be carried out without the use of a laminar flow cabinet. This new method was named as the CSUP technique. When glassware is sterilized by rinsing with sodium hypochlorite solution and the culture medium is poured into the glassware, the medium becomes sterilized and even the transferring of axenic cultures can be performed without the use of a laminar flow cabinet. Hence the use of this method saves the capital cost by Rs. 1.5 – 2.0 Million, which will encourage entrepreneurs to have small-scale laboratories either as an extra income earner or develop their own varieties as in the case of orchid growers. This study was undertaken with the objective of evaluating the feasibility of the CSUP technique to replace the use of autoclave and the laminar flow cabinet used in *in vitro* micropropagation of plants to create an aseptic environment and culture media.

METHODS AND MATERIALS

The experiments were conducted in the Tissue Culture Laboratory, Department of Crop Science, Faculty of Agriculture, University of Peradeniya, Peradeniya.

Explant materials*: In vitro* micrpropagated *Anthurium andreanum* Lind. cv ' Tropical Red" shoots of 3 cm were used as explant material for this experiment. They were subcultured several times in hormone-free medium prior to use in the experiment so as to reduce the hormonal effects. Twenty (20) randomly selected shoots of uniform size were used in each treatment, which included 5 shoots per glass jar.

Preparation of the sterilizing solution: Four concentrations, 5, 10, 15, and 20% were prepared on volume to volume basis with distilled water using 5.02% sodium hypochlorite (Clorox™), commercially available bleach.

CSUP Technique for Sterilization of glassware: Glass jars were cleaned by washing with liquid soap (Teepol®-Lankem™), followed by rinsing with a disinfectant such as Dettol™ and drying in an oven of 65 ^{0}C. A plastic tray was rinsed with 5% sodium hypochlorite solution. Then the glass jars were immersed in 500 mL of the same solution, in a plastic bowl by placing them horizontally and rotated several times. Subsequently, the jars were placed upside down in the solution to immerse the rims in the solution and rotated several times to wash thoroughly. The jars were next placed upside down on the plastic tray and kept for 10 min. Lids of these jars were also rinsed by dipping in the same sodium hypochlorite solution and placed on the tray with the jars. Four jars and lids were rinsed using each concentration of sodium hypochlorite.

Preparation and sterilization of the culture-growing medium: Murashige and Skoog (MS) medium (1962) was used throughout the experiment. Full strength MS medium was supplemented with 3% sugar, 100 mg/L Myo-inositol, 0.5 mg/L benzyle amino purine (BAP) and 15% (v/v) coconut water. The pH of the medium was adjusted to 5.8. Phytagel (0.46% w/v) was added and boiled until the gel dissolved. The medium (30 mL each) was poured into glass jars, which were sterilized with sodium hypochlorite and covered with lids. This procedure was followed for all four treatments (4 sodium hypochlorite concentrations) except the control. For the control, the same quantity of culture medium with the same composition was poured into glass jars and autoclaved at a temperature of 121 ˚C and a pressure of 1.05 kg/cm^2 for 20 min.

Establishment of cultures: Establishment of cultures was carried out in a glass cage (90 x 90 x 60 cm) placed in a room, which was free from dust. The glass cage was wiped with 70% ethanol and a spirit lamp was lit 20 minutes prior to culturing. Five (05) randomly selected *in vitro* anthurium shoots of cv 'Tropical Red' with approximately the same weight were transferred on to the medium contained in each glass jar randomly, using four jars per treatment in all four treatments. Next the culture jars were covered with a clean sheet (not autoclaved) of cellophane. Culturing in the control treatment (autoclaved medium) was carried out in a laminar flow cabinet. All cultures were maintained at 23 -25 °C temperature under 16/8 h (day/night) photoperiod with a light intensity of approximately 1500 lux throughout the experiment. Jars were arranged according to a complete randomized design. There were four replicates for each treatment. Experiment was repeated three times.

Observations: Number of contamination free treatments, number of newly produced shoots per treatment, fresh

weight of 5 plantlets in each treatment and the dry weight of shoots were recorded after 6 wks. Five plants from each treatment were randomly selected to measure the fresh weights and dry weights. Initial dry weights were calculated with oven drying (at 60 ^{0}C) of a reference sample and then estimating the coefficient of drying.

Data analysis: Effect of different concentrations of sodium hypochlorite on the percentage of contamination free cultures were analyzed using Proc Probit procedure in SAS package. Dry weight increases were analyzed by one way ANOVA test with MINITAB. The effects on shoot development were analyzed by using Kruskal-Wallis test with MINITAB.

RESULTS

Effects of the use of different concentrations of sodium hypochlorite solution for sterilization

The percentage of contamination free cultures varied from 72 to 78 % in the four treatments with different concentrations of sodium hypochlorite. The autoclaved medium had 73% contamination free cultures. There was no significant difference ($p > 0.05$) in the percentages of contamination free cultures among treatments with different concentrations of sodium hypochlorite solution, and the autoclaved cultures where a laminar air flow cabinet was used to transfer cultures (Table 01). These results showed that the CSUP technique is as effective as the combination of autoclaving and the use of a laminar flow cabinet in creating an aseptic environment for plants to grow under *in vitro* conditions. The results also indicate that sodium hypochlorite concentrations 5, 10, 15 and 20% used in this study are equally effective in sterilizing the medium and the glass jars. Therefore, the lowest concentration can be used as a cost effective measure.

Effects of the use of different concentrations of sodium hypochlorite for sterilization on dry weight increase of shoots

The highest dry weight gain was seen in the shoots, where sodium hypochlorite solution of 10% (v/v) concentration was used for sterilization (Figure 01). There was no significant difference in dry weight increase of shoots among treatments with different concentrations ($p > 0.05$). Also there was no significant difference between the treatments and the control. As indicated by this study, the method of sterilization of the culture medium does not affect the plant growth. Though it is not significantly different, the results show that the dry weight gain is the lowest in the culture medium sterilized using the autoclave. Based on the results, 5% (v/v) sodium hypochlorite solution can be used effectively, for sterilization of culture medium in terms of overcoming contaminations and without affecting dry weight increase, since it reduces cost of production.

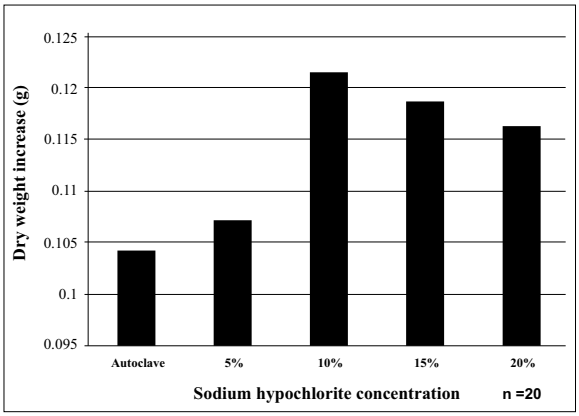

Figure 01: Dry weight increase of anthurium cv "Tropical Red" shoots where four sodium hypochlorite concentrations were used for sterilization

Table 01: Effect of sterilization using different sodium hypochlorite concentrations on contamination of cultures

Treatment no.	Sodium hypochlorite concentration %	Contamination free cultures %
1	Autoclaved (control)	73
2	5	78
3	10	73
4	15	78
5	20	72
		n = 20

Table 02: Shoot multiplication of anthurium cv 'Tropical Red' using four sodium hypochlorite concentrations for sterilization

Treatment no.	Sodium hypochlorite concentration %	Number of shoots
1	Autoclaved (control)	20.40
2	5	21.66
3	10	22.86
4	15	24.05
5	20	24.65
		n = 20

Effect of the use of different concentrations of sodium hypochlorite for sterilization on formation of shoots

Table 2 shows that the shoot cultured in each jar produced more than 20 shoots regardless of the concentration of sodium hypochlorite used for sterilization. The highest number of shoots (24.65) was observed in the jars sterilized with 20% (v/v) sodium hypochlorite. The analysis carried out by Kruskal-Wallis test with MINITAB shows that there is no significant difference between the treatments and the control ($p > 0.05$) although the number of shoots increased with the use of increasing concentrations of sodium hypochlorite. It was found that, there was no correlation between shoot formation and the concentration of sodium hypochlorite solution used for sterilization. The lowest number of shoots was obsered in the control.

DISCUSSION

The results of this study reveals that sodium hypochlorite solution even at a low concentration such as 5% (v/v) can result in an aseptic environment in the glass jar and culture medium. Once the glass jar is rinsed with sodium hypochlorite solution and allowed to drain for several minutes, it forms a thin chemical film on the inner surface of the jar. After the medium solidifies in the jar it is possible that a film may have also formed on the medium. Hence, there is a protective film of sodium hypochlorite preventing microorganism growth when they come in contact with the medium during the culturing process even in the absence of an aseptic environment created by the laminar flow cabinet. Furthermore, when the medium is disturbed by the cut end of the shoot during culturing, the solution may defuse into the medium around the shoot. Thus sodium hypochlorite solution covers the exposed surfaces preventing microorganism growth. Sodium hypochlorite degrades within a few days but as the jar is closed it remains aseptic. Due to quick degradation, sodium hypochlorite does not become toxic to tender shoots. Vitamin B and some plant growth hormones in the culture medium are heat labile and can degrade easily during autoclaving. In the CSUP technique, such undesirable effects can be avoided and due to quick degradation of sodium hypochlorite it does not affect the activity of hormones and vitamins. This may be the reason for the performance of shoot growth and multiplication in CSUP technique, which was not significantly different from the media sterilized through autoclaving in this study.

Sodium hypochlorite is a universal disinfectant, which is pH sensitive, has a short shelf life when diluted, is easily neutralized by organic material, is corrosive and is an oxidizing product. The commercial products of sodium hypochlorite normally contain 5.05% of NaOCl as the active ingredient. Sodium hypochlorite in water produces hypochlorous acid (HOCl) and hypochlorite ions (OCl^-) (Estrela *et al*., 2002). The molecular form of hypochlorous acid (HOCl) is the active form of bleach, which destroys microorganisms (Ushijima & Nakano, 1980). HOCl penetrates slimy layers, cell walls and protective layers of microorganisms and effectively kills pathogens as a result. The microorganisms will either die or suffer from reproductive failures (Leifert & Waites, 1994). Hypochlorite ion (OCl^-) is also an active form but very weak and slow to destroy microorganisms (*www.poolcenter.com/chlor.htm*.). HOCl is a neutral, much more reactive and stronger disinfectant than OCl^-, as HOCl is split into hydrochloric acid (HCl) and atomic Oxygen (O). Oxygen is also a very powerful disinfectant (*www.lenntech.com*). Dust particles containing microorganisms get deposited on surfaces continuously. These microorganisms live several hours and die if a favourable growth medium is not found. During transfer of cultures they come in contact with culture media, which are rich in sources that encourage their vigorous growth. The dust particles that get deposited on sodium hypochlorite treated glass jars and the dust particles that fall onto the growth medium during culturing are destroyed by both HOCl and OCl^- in the film of sodium hypochlorite formed on the inner wall of the glass jar in the CSUP technique.

Deionized water should be used to prepare bleach solution in order to keep its strength as effective as possible, because the presence of transition metal (Cu, Fe and Ni) ions is known to catalyze the decomposition of liquid bleach, contributing to the loss of bleach strength and the formation of oxygen.

Temperatures around 25 to 30 ^{0}C increase the bleaching activity, however, temperatures above that may degrade the chemical. Relatively cold temperature reduces its activity and therefore the chemical can be stored in a refrigerator for several months.

Although, the use of higher concentrations of sodium hypochlorite leads to lesser degrees of contamination of plant cultures, they can affect plant performances *in vitro*. This study reveals that 5% (v/v) sodium hypochlorite can be effectively used in *in vitro* micropropagation as an alternative to the use of the autoclave and the laminar flow cabinet. It is advisable for the beginners to tissue culture to use a higher concentration (10 or 15% v/v) and reduce it over time with experience. Although

Table 03: Cost comparison of conventional method with CSUP technique for preparation and handling of one liter (1L) of Murashige and Skoog (MS) medium (approximate calculation)

Conventional tissue culture	Cost (Rs.)	CSUP technique	Cost (Rs.)
Equipment:		Equipment:	
Autoclave	1500,000.00	Hot plate	600.00
Laminar flow cabinet	600,000.00	Glass hood	1200.00
Microwave oven / oven	40,000.00	Spirit lamp	150.00
Steribead sterilizer	50,000.00	Culture jars	250.00
Culture jars	250.00	Plastic tray and cup	150.00
pH meter	60,000.00	pH meter	60,000.00
Initial cost (except laboratory and other equipment)	**2,250,250.00**	**Initial cost (except laboratory and other equipment)**	**62,350.00**
Consumables:		Consumables:	
MS medium	115.00	Isopropyl alcohol	90.00
Agar	110.00	MS medium	115.00
Electricity for autoclave	40.00	Agar	110.00
Electricity for laminar flow cabinet	60.00	CSUP solution (5%)	12.50
Electricity for steribead sterilizer	50.00	Electricity for others	50.00
Electricity for others	50.00	Plant growth regulators	50.00
Isopropyl alcohol	90.00	Miscellaneous	100.00
Plant growth regulators	50.00		
Miscellaneous	100.00		
Cost for preparation of 1L medium	**665.00**	**Cost for preparation of 1L medium**	**527.50**
Total	2,250,915.00	Total	62,877.50

this study concentrated on *in vitro* multiplication, this technique can also be applied to the pre-transplant stage as growth of the shoots are not affected. The CSUP technique was applied recently to regenerate anthurium plants *in vitro* using leaf pieces as the explant and it showed that lower concentrations, 5 and 10 % of sodium hypochlorite, produced successful results (*S.N. Dammullage*, *unpublished data*).

CSUP method, in contrast to the conventional method, can save approximately 97% of the equipment cost (Table 03). This figure was derived at by replacing the minimal requirement of one autoclave and one laminar flow cabinet as the most expensive pieces of equipment for a tissue culture laboratory. Moreover, the ability to use the medium for culturing in the same day it is prepared, is another advantage.

At present, CSUP technique is successfully carried out by one commercial grower, Ramya Horticulture Pvt. Ltd., Walpita (*Personal communication, 5th January 2011*) to produce anthurium planting material in large quantities and in several small scale laboratories. When sterilizing a large amount of glassware, 500 mL of diluted sodium hypochlorite solution can be used to rinse 50 glass jars, their lids and trays. As sodium hypochlorite is corrosive and emits chlorine gas, it is advisable to use a pair of gloves, a mask and a pair of safety glasses for the protection of the individuals undertaking the CSUP technique. If this technique is being used throughout the day it is recommended to use a fume hood or an exhaust fan to expel the chlorine gas.

CONCLUSION

The study reveals that the use of 5% (or higher) dilution of sodium hypochlorite, (common house hold bleaching solution) can effectively replace the use of two expensive pieces of equipment, the autoclave and the laminar flow cabinet, in *in vitro* multiplication of anthurium cv 'Tropical Red'.

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