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SHORT COMMUNICATION

A PROCEDURE FOR STERILISING LEAF EXPLANTS COLLECTED FROM WILD NEEM *(AZADIRACHTA INDICA)* TREES

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Abstract : Experiments conducted to find suitable sterilisation procedures for neem explants collected from trees growing in the wild showed that they could not be sterilised by the method for leaves collected from the trees reared in the laboratory (10% NaOCl for 10 min.) but required more rigorous sterilisation methods. Leaf explants collected from the trees growing in the wild could not be sterilised using different concentrations of NaOCl (10%, 15% 20% & 25%), $HgCl_2$ (0.1%, 0.5%, & 1%) for different time intervals (5, 10, 15, 20 and 30 mins) but could be sterilised by combining $HgCl_2$ and NaOCl (0.1% $HgCl_2$ for 5 min followed by 10% NaOCl for 10 min). Leaf damage was observed when 70% ethanol was used as a pre-treatment.

Key Words: *Azadirachta indica*, leaf explants, sterilization.

INTRODUCTION

Azadirachtin, which is extracted from neem seeds, shows insect antifeedant as well as growth regulatory activity against a wide range of insect pests.[1] It has been shown that azadirachtin is biodegradable without having residual effects to the environment[2] and therefore, can be used as an environmentally friendly insecticide in crop protection programmes.

However, the low quantities of azadirachtin present in neem seeds and the expense of purification makes the pure compound very expensive (15,000 U.S. $ per kg). Therefore, the chemical synthesis[2] of azadirachtin and production using plant cell culture techniques [3-6] have been attempted.

Maximisation of product synthesis is desirable when plant cell culture techniques are applied to produce valuable secondary metabolites. Strategies for producing high yielding cell cultures include, establishment of cell lines from selected high yielding trees, followed by clonal multiplication and optimisation of culture conditions.[7] Therefore, it is very important to establish cell lines from high producing

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wild growing neem trees (referred as wild neem) when cell culture techniques are used to produce azadirachtin. Kearney *et al.*, (1994)[3] reported successful callus initiation from neem leaf explants which was subsequently shown to be successful irrespective of the country of the origin of the plant materials by Eeswara *et al.*,(1997).[6] Even though, the medium was successful in initiating calli, high rate of contamination of cultures were seen when leaf explants collected from wild growing neem were sterilised by the method (i.e. 10% NaOCl for 10 min) used for laboratory grown materials. Therefore, investigations were carried out to find techniques suitable for sterilisation of neem leaf explants from wild trees.

METHODS AND MATERIALS

Leaf explants collected from a wild neem tree at University experimental station at Dodangolla, which is 15 km away from the University of Peradeniya were used for the experiments. Immature leaves (slightly reddish colour) were picked directly from the tree and sprayed with water before packing in polythene bags which were placed in a water bucket during the transport (1 hour) from the experimental station to the tissue culture laboratory at University of Peradeniya. The leaves were thoroughly washed with tap water followed by mild liquid soap (Teepol C.I.C, Sri Lanka) and sterilised distilled water (pre-treatment) as soon as they were taken to the laboratory, prior to receiving further specific treatments. After this stage all the treatments (experiments 1 to 6, Table 1) were done under clean environmental conditions in a laminar flow cabinet. Treated leaf explants were thoroughly washed with sterile distilled water in between each treatment in each experiment and prior to being incubated at 25 ^{0}C in the dark on Murashige and Skoog medium containing 1 mg l^{-1} BAP and 4 mg l^{-1} IBA (Maintenance Medium).[3] Each treatment was replicated 30 times. In addition, leaf explants treated under different sterilisation regimes were kept in Petri dishes containing glucose Agar and nutrient Agar (Oxoid, UK) to observe any microbial contamination. The number of cultures without contamination was recorded. Results were subjected to Chi-squared analysis after categorisation into cultures, which contaminated and those, which were not. The experiment 6 was conducted to replace the Bleach with standard chemicals (NaOCl, Sigma, UK). Pre-treated leaf explants were first sterilised with 0.1% $HgCl_2$ for 5 min and thereafter, leaves were surface sterilised with three different concentrations of NaOCl(Sigma UK) for 10 minutes (Table 1). Leaf explants sterilised with 10% commercially available NaOCl (Bleach, Care product) in addition to 0.1% $HgCl_2$ treatment were used as the control (Table 1).

The success of sterilisation procedure established based on the results of experiments 1 to 6 was further investigated using neem leaf explants collected from the 15 wild neem trees from four locations (Dambulla, Kithalawa, Maha-Illuppallama and Kalawewa) in two agro-ecological zones (dry and intermediate) in Sri Lanka. Leaves were picked directly from trees, and transported (4 hours) to University of Peradeniya as described for the leaves from tree at Dodangolla farm. Thereafter,

Table 1 : Effect of 70% Ethanol, Bleach[1], NaOCl (Sigma, UK) and $HgCl_2$ on sterilisation of wild neem leaf explants.

Effect of 10% Bleach, ethanol and Tween 20												
Treatment code	1A	1B	1C	1D	1E	1F	1G	1H				
1. 70% Ethanol treament (3 minutes)	*	*	*	*	-	-	-	-				
2. Tween Twenty[2]	*	*	-	-	*	*	-	-				
3. 10% Bleach treatment (%v/v)	*	*	*	*	*	*	*	*				
4. Duration of Bleach treament (minutes)	10	20	10	20	10	20	10	20				
5. % Survival	0	0	0	0	0	0	0	0				
Effect of Bleach Concentration and Time Duration												
Treatment code	2A	2B	2C	2D	2E	2F	2G	2H	2I	2J	2K	2L
1. 70% Ethanol treatment (3 minutes)	*	*	*	*	*	*	*	*	*	*	*	*
2. Tween Twenty[2]	*	*	*	*	*	*	*	*	*	*	*	*
3. Concentration of Bleach treatment (%v/v)	10	10	10	15	15	15	20	20	20	25	25	25
4. Duration of Bleach treatment (minutes)	10	20	30	10	20	30	10	20	30	10	20	30
5. % Survival	0	0	0	0	0	0	0	0	0	0	0	0
Effect of 0.1% $HgCl_2$ and Time Duration												
Treatment code	3A	3B	3C	3D	3E	3F	3G	3H	3I	3J		
1. 70% Ethanol treatment (3 minutes)	*	*	*	*	*	-	-	-	-	-		
2. 0.1% $HgCl_2$, $HgCl_2$ treatment (w/v)	*	*	*	*	-	*	*	*	*	-		
3. Duration of $HgCl_2$ treatment (minutes)	5	10	15	20	20	5	10	15	20	20		
4. % Survival	0	3.3	0	6.7	10	0	6.7	3.3	0	6.7		
Effect of different concentrations of $HgCl_2$ and Time Duration												
Treatment code	4A	4B	4C	4D	4E	4F	4G	4H	4I	4J	4K	4L
1. 70% Ethanol treatment (3 minutes)	*	*	*	*	*	*	*	*	*	*	*	*
2. Concentration of $HgCl_2$ treatment (w/v)	0.1	0.1	0.1	0.1	0.5	0.5	0.5	0.5	1.0	1.0	1.0	1.0
3. Duration of $HgCl_2$ treatment (minutes)	5	10	15	20	5	10	15	20	5	10	15	20
4. % Survival	3.3	6.7	3.3	10	10	13	6.7	17	20	17	23	6.7
Combined effect of $HgCl_2$ and Bleach												
Treatment code	5A	5B	5C	5D	5E	5F	5G	5H	5I	5J		
1. 70% Ethanol treatment (3 minutes)	*	*	*	*	*	-	-	-	-	-		
2. Tween Twenty	*	*	*	*	*	*	*	*	*	*		
3. 10% Bleach treatment (%v/v) for 10 minutes	*	*	*	*	*	*	*	*	*	*		
4. Concentration of $HgCl_2$ treatment (w/v)	0.1	0.1	0.1	0.1	0	0.1	0.1	0.1	0.1	0.1		
5. Duration of $HgCl_2$ treatment (Minutes)	5	10	15	20	20	5	10	15	20	20		
6. % Survival	100	100	66.7	100	20	80	53.3	60	66.7	26.7		
Replacement of Commercially available Bleach with Standard Chemicals (NaOCl, Sigma,UK)												
Treatment code	6A	6B	6C	6D								
1. 0.1% $HgCl_2$ treatment (w/v) for 5 minutes	*	*	*	*								
2. 5% NaOCl (Sigma,UK) for 10 minutes	*	-	-	-								
3. 10% NaOCl (Sigma, UK) for 10 minutes	-	*	-	-								
4. 15% NaOCl (Sigma, UK) for 10 minutes	-	-	*	-								
5. 10% Bleach for 10 minutes	-	-	-	*								
6. % Survival	55	75	85	45								

[1] Commercially available NaOCl (Bleach, Care Products Sri Lanka)

[2] Tween Twenty was incorporated into Bleach Solutions

* Indicate the presence of specific treatment

pre-treated leaves were surface sterilised with 0.1% $HgCl_2$ for 5 minutes followed by 10% NaOCl for 10 minutes and were cultured on Maintenance Medium.

RESULTS AND DISCUSSION

Immature leaves were selected since young tissues are often found to be free of detectable internal population of viroids, viruses, mycoplasms, bacteria and fungi.[8] It was found that sterilisation of neem leaf explants collected from wild growing trees was unsuccessful (100% contamination) when they were treated with 10% Bleach alone or following exposure to 70% ethanol for 3 min (Table 1). Furthermore, leaf explants treated with concentrations of Bleach of up to 25 % for up to 30 minutes also showed 100% contamination (Table 1). After these treatments leaf explants cultured on Glucose and Nutrient Agar showed very high levels of both bacterial and fungal contamination.

Very few leaf explants treated with 0.1% $HgCl_2$ were observed to be free of contamination and the results were not significant at the 5% probability level (Table 1). Exposure to a variety of concentrations of $HgCl_2$ for different time intervals, also produced few cultures without contamination (Table 1) but results were not significantly different. Leaf explants treated with $HgCl_2$ and cultured on Glucose Agar and Nutrient Agar showed more fungal than bacterial contamination. These results clearly showed that sterilisation of wild neem leaf explants could not be achieved following the procedure for laboratory-grown materials[3]. Taking explants from other field-grown plants has previously been shown to reduce the efficiency of the subsequent disinfection process.[8] Furthermore, wild neem leaf explants could not be surface sterilised using either NaOCl or $HgCl_2$ alone.

The effects of different concentrations of $HgCl_2$ combined with 10% Bleach solution on the sterilisation of wild neem leaf explants are shown in Fig 1. In this experiment very low levels of contamination were observed where leaf explants were treated with 70% ethanol prior to being subjected to $HgCl_2$ and 10% Bleach treatments. However, leaf explants treated with 70% ethanol became brown in colour and callus production from uncontaminated leaf explants was very low (Fig. 1). Chi-squared analysis showed a highly significant effect of ethanol on production of brownish leaf explants ($p<0.001$). The use of ethanol as a pre-treatment may cause the penetration of NaOCl and $HgCl_2$ into internal tissues and consequently damage the leaf explants. Further statistical analysis conducted only on those leaf explants, which had not been treated with 70% ethanol, showed significant differences ($p<0.05$). This was due to the high contamination % observed in treatment 5J, in which leaf discs had only been treated with 10 % Bleach. There were no significant differences at 5% level for the other four treatments (5F, 5G, 5H & 5I). Therefore, treatment 5F containing the lowest concentration of $HgCl_2$ (0.1% $HgCl_2$ for 5 min followed by 10% Bleach for 10 min) was used for further work.

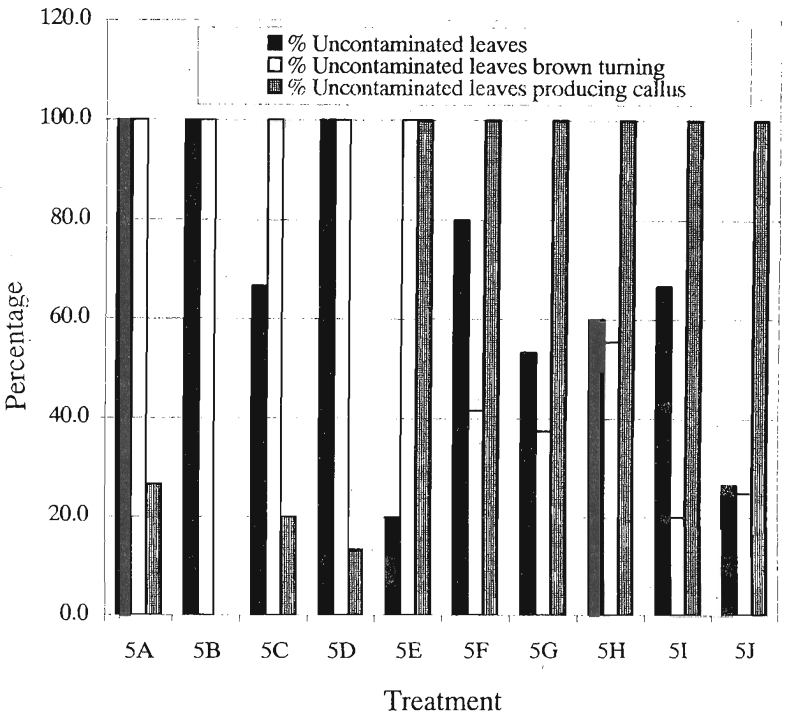

Figure 1: Effect of different concentrations of $HgCl_2$ in combination with 70% Ethanol and 10% NaOCl on survival, browning and callus production on wild neem leaf explants.

The results of the experiment, which was conducted to compare the commercially available Bleach with standard chemicals are shown in Table 1. Chi-squared analysis showed significant differences ($p \leq 0.01$) between 10% Bleach and all the other treatments except for 5% NaOCl. There was no significant difference ($p \leq 0.05$) between 10% NaOCl and 15% NaOCl. At the end of the sixth week period the amount of callus produced was measured and one-way analysis of variance showed no significant differences for the 10% Bleach and 10% NaOCl (Sigma, UK) treatments.

The surface sterilisation procedure described (0.1% $HgCl_2$ for 5 min followed by 10% NaOCl (Sigma, UK) for 10 min) was effective in 85% of explants but this was found to be acceptable at practical level. Leaf explants collected from 15 wild neem trees from four locations (Dambulla, Kithalawa, Maha-Illuppallama and Kalawewa) in two agro-ecological zones (Dry zone and Intermediate zone), could be successfully sterilised by applying this method.[6] Callus was successfully initiated using the maintenance medium, in accord with the earlier results[3], irrespective of differences in stock plant origin. Callus was brown in colour and contained hard, partially differentiated, callus clumps, which became white and friable with continuous subculturing. The present study showed that by combining two

disinfectants successful surface sterilisation could be achieved. Disinfection with $HgCl_2$ was reported to be practically useful when the presence of fungal contaminants made disinfection by hypochlorite ineffective.[9] Thus, $HgCl_2$ may kill the micro-organisms, which cannot be killed by NaOCl and *vice versa*.

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