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Explants Sterilization Protocol for *In-vitro* Propagation of Elite Enset (*Ensete ventricosum* (Welw.) Chessman) Cultivars

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ABSTRACT

Previous Enset in vitro micropropagation attempts were hindered by explants microbial contamination and for successful in vitro propagation explants specific standard sterilization protocol have to be developed. Therefore, this study was designed to standardized sterilization protocol for shoot tip and leaf sheath explants of three elite Enset (Ensete ventricosum (Welw.) Chessman) cultivar. Sodium hypochlorite under different concentrations (1, 2 and 3% in double sterilization for shoot tip explants and 0.5, 1 and 2% for 10 min immersion for leaf sheath explants) was used for surface sterilization of explants. Explants sterilized with 70% ethanol without NaOCl was used as control. Sterilized explants were cultured on 500 mg/L cefotaxim containing MS medium to evaluate response of explants sterilization to different NaOCl concentration. Results of shoot tip sterilization experiment showed that 2% NaOCl was found to be significantly superior compared to control, 1 and 3% concentration at $p<0.05$ for obtaining contamination free and growing explants. Among leaf sheath explants sterilization treatments, 1% NaOCl was 100% effective for cultivar Yanbule, while 0.5% NaOCl was found to be best with regard to achieving highest percentage of contamination free healthy leaf sheath culture of Mesena and Endale cultivars.

Keywords: Enset, Sterilization, Leaf sheath, *NaOCl*, Shoot tip

INTRODUCTION

Enset (*Ensete ventricosum* (Welw.) Chessman) is a perennial, herbaceous plant belonging to the Musacea family and in the genus Ensete [1]. Enset grows wild or is used as ornamental plant in many part of the world. Its cultivation and use as economic crop and food securing crop is, however, restricted to Ethiopia [1-3], where it serve as a staple and co-staple food. Estimated more than 20 percent of Ethiopia's population that live in the highlands of the southern Ethiopia depend on enset for food (in the form of Qocho and Bula), fiber, animal forage, construction materials and medicines and its demand is increasing throughout the country particularly as source of food and fiber for industry [4].

Ethiopia, being a food insecure country and in protracted food crisis [5] due to climate change and others, enset can improve food security in all areas including drought-prone regions. The crop is drought resilient and gives higher yield per unit area which can sustain a large population per unit area compared to other crops [6]. However, the crop coverage as well as productivity under enset is declining from time to time due to bacterial wilt and others several threats to the crop [7]. Conventionally enset is propagated by vegetative means through suckers obtained from 4-6 years old decapitated and decorticated mother corm [3,8,9]. Therefore, the current conventional enset propagation method is at expense of planting suckers from matured enset corm, which is time consuming and has various limitations such as transmission of the deadly bacterial wilt disease and other problems.

On top of these, the sucker's multiplication rate is too low, it takes many years to scale up the cultivars of preference for production. Currently enset cultivation is heavily threatened by bacterial wilt problem which is caused by *Xanthomonas*

campestris pathovar (pv) musacearum transmitted by various means but the most easy way being the conventional vegetative propagation causing wide spread problem of the disease [10,11]. Hence, *in-vitro* mass propagation is recommended to assist as well as overcome the shortcomings of the conventional method through provision of clean planting material. However, successful *in-vitro* propagation requires following serious of procedure; among these, appropriate explants selection and effective explants sterilization are the most critical pre-requisite steps for successful *in-vitro* propagation. Basically, explants sterilization is the process of making explants contamination free before establishment of cultures. It is important that the explants be free of any contaminants including endophytic prior to tissue culture without losing their biological activity [12,13].

Various sterilization agents are used to decontaminate plant tissues for in vitro cultures. These sterilants are also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explants to various sterilants, the sequences of using these sterilants has to be standardized to minimize explants injury and to increase survival rate while ensuring effective sterilization [14]. In essence, requirements on the type, concentration and time of exposure differ from one plant to another and for different tissue of plant depending on their morphological characters like softness or hardness of the tissue [15].

In-vitro regeneration and propagation of enset highly inhibited due to microbial contamination [16,17]. Therefore, the present study was designed to develop effective explants sterilization protocol for shoot tip and leaf sheath explants of three elite enset cultivars, using antibiotic Cefotaxim to the medium to decrease explants endophytic contamination, ethanol and *NaOCl* by varying its concentration in order to minimize explants surface contamination rate by reducing explants injury due to chemical toxicity. So far this is the first report utilizing different concentration of *NaOCl* for shoot tip and leaf sheath explants sterilization of enset.

MATERIALS AND METHODS

Experimental site and source of experimental material

The experiment was conducted in National Agricultural Biotechnology Research Center (NABRC) at Holetta, Ethiopia; from July 2016 – January 2017. Three enset cultivars, namely Yanbule, Mesena and Endale were obtained from Areka Agricultural Research Center of Ethiopia and were grown at Holetta NABRC green house. These cultivars were registered, certified and nationally released from Areka Agricultural Research Centre based on their early maturing ability and high Qocho production potential [18,19].

Medium preparation

After preparing MS and plant growth regulators (PGRs) stock solutions and appropriate mixing, the pH of the medium was adjusted at 5.8 and autoclaved at a temperature of 121^{o}C with a pressure of 103 Kpa for 20 min [20]. MS basal medium was enriched with 30 g/L Sucrose, 1.0 g/L Active Charcoal, 4.5 g/L agar, and 500 mg/L filter sterilized cefotaxime and supplemented with 1.5 mg/L of BAP for all shoot tip explants culture, while for leaf sheath explants culture MS basal medium enriched with 2 mg/L 2, 4-D in combination with 0.25 mg/L of BAP. After autoclaving, the culture medium was stored in a clean dust free chamber for 4-5 days before use in order to check for any media contamination.

Explants preparation and surface sterilization

Two different types of explants (shoot tip and leaf sheath) were used in this sterilization experiment. Suckers of explants source were carefully removed, without damaging the rhizome portion and brought to the preparation room in the lab. To produce shoot tip explants, the roots and outer tissues of the suckers were removed until the shoot measured to about 3.0 cm in length and 3.0 cm width at the base with the help of a sharp knife (Figure 1A). To produce leaf sheath explants, the 2nd leaf sheath from the inner side, were collected.

During pre-sterilization, shoot tip and leaf sheath of stock tissue was rinsed with in absolute ethanol for 3 min and both explants were washed thoroughly under running tap water with Largo and Tween 20 with slight shaking and washed until largo and Tween 20 removed. The Shoot tip and leaf sheath explants were then dipped in 70% (v/v) ethanol for 10 min and 3 min respectively in aseptic condition in the laminar air flow hood. The shoot tip explants were then first treated with 1, 2 or 3% Sodium hypochlorite *(NaOCl*) with 3 drops of Tween 20 for 20 min. But, leaf sheath explants aseptically sterilized with 0.5, 1 or 2% of *NaOCl* for 10 min. Thereafter, the outer surface of the explants that was exposed to sterilizing agent was removed and the explants were trimmed from all sides. Disinfection of shoot tip explants was done again in the same way using *NaOCl* for 10 min to make double sterilization. Both type of explants

were then rinsed with sterile distilled water 3 times and shoot tip explants trimmed to have final size of about 0.8-1cm length and 0.5-1 cm diameter with its subtending corm and leaf sheath explants adjusted about 0.25 cm^2 final sizes. Explants treated with 70% ethanol without *NaOCl* were used as controls for both types of explants. Then after all shoot tip and leaf sheath explants were cultured on respective MS medium, in order to minimize explants phenol oxidation culture were preserved at dark growth room without any illumination for two successive weeks. Then after, culture transferred to light condition at 25 ± 20°C and 16 h photoperiod of white fluorescent light of (20 µ mol/m^2/s) intensity. The experiment conducted with four replications and each shoot tip and leaf sheath sterilization treatment was contained 16 explants.

Experimental design and data analysis

The experiments were conducted in a Completely Randomized Design (CRD). Data on percentage of explants that were contamination free and survived during sterilization were subjected to Analysis of Variance (ANOVA) and significant differences among treatments were compared by Duncan's multiple range tests at 5% level of significance by using SAS software.

RESULTS AND DISCUSSION

Sterilization of shoot tip explants

Analysis of variance (ANOVA) revealed that concentration of *NaOCl* and its interaction with cultivars had significant difference ($P<0.05$) in overcoming contamination and improving survival of shoot tip explants. Compared to the control, application of *NaOCl* for sterilization of explants gave significantly reduced contamination levels and improved survival rate of explants in culture (Table 1).

Table 1: Effect of *NaOCl* concentration on explants sterilization

Cultivars	NaOCl (% v/v)		Explants immersion time(min) in NaOCl in double sterilization	Mean No of alive and Contamination free explants/ treatment	Percentage of alive and Contamination free explants (%) (mean ± SD)
Mesena	0		20 and 10	0	0.0 ± 0.0
	1		20 and 10	2	50.0 ± 25.0
	2		20 and 10	4	**100 ± 0.0**
	3		20 and 10	3.33	83.3 ± 14.4
Yanbule	0		20 and 10	0	0.0 ± 0.0
	1		20 and 10	1.33	33.3 ±14.4
	2		20 and 10	4	**100 ± 0.0**
	3		20 and 10	3.67	91.7 ± 8.3
Endale	0		20 and 10	0	0.0 ± 0.0
	1		20 and 10	2.33	58.3 ± 14.4
	2		20 and 10	4	**100 ± 0.0**
	3		20 and 10	3	75.0 ± 0.0
CV					18.24
ISL					*

CV=Coefficient of Variation, SD=Standard Deviation, ISL=Interaction Significance Levels, *=Significant, Data represent the mean of four replicates. In a column, means followed by the same letter are not significantly different at the 5% level by DMRT.

Sodium hypochlorite is the most commonly used disinfectant for surface sterilization of different type of explants [16,21,22]. Most shoot tip explants sterilized with control treatment (Sterilization without *NaOCl*) were contaminated after 10 days, thus giving 0% contamination free cultures for the control group by day 23 for all tested cultivars. During late time of culture some explants started to show symptoms of indigenous contamination due to endophytes while surface contaminant quickly developed the bacterial colonies around the bases of explants which causing a complete loss of cultures (Figure 1B). Although 1% *NaOCl* resulted in greater percentage of contamination free and survived shoot tip explants, but values were significantly lower than that of 2 and 3% *NaOCl* in all cultivars. In this study, the potential for *NaOCl* to result in high percentage of contamination free and live explants was in an order of 2>3>1%, suggesting that 2% *NaOCl* is optimal concentration for effective shoot tip sterilization (100%) of the three tested cultivars of enset (Figure 1C). But there had not been significant difference when compared to 3% for the case of cultivar Yanbule.

On over all bases, all the three cultivars had almost the same response to different concentration of *NaOCl*. However, the present data showed that with increase in the *NaOCl* concentration, a decrease in contamination and explants survival rate was observed.

The percent of live and contamination free explants increased from 1 to 2% *NaOCl* beyond which level results show less contamination but increased death of explants. This could be due to the phytotoxic effect of *NaOCl* at higher concentration. During sterilization, the explants should not lose their biological activity and only contaminants should be eliminated [13]. Therefore, the present study showed that surface sterilization experiment was effective when shoot tip explants were treated with 70% ethanol for 10 min followed by double sterilization with 2% of *NaOCl* first for 20 min and then 10 min.

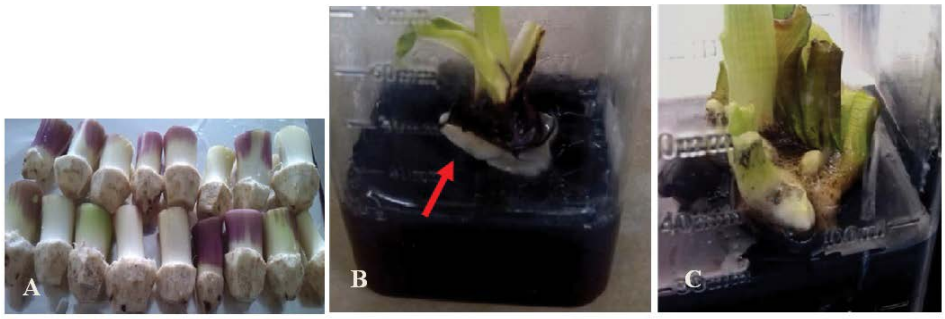

Figure 1: Shoot tip explants sterilization study. Figure 1A: Shoot tip explants during pre-sterilization; Figure 1B: Contaminated shoot tip culture sterilized without *NaOCl*; Figure 1C: Contamination free initiated shoot tip culture sterilized with 2% *NaOCl*.

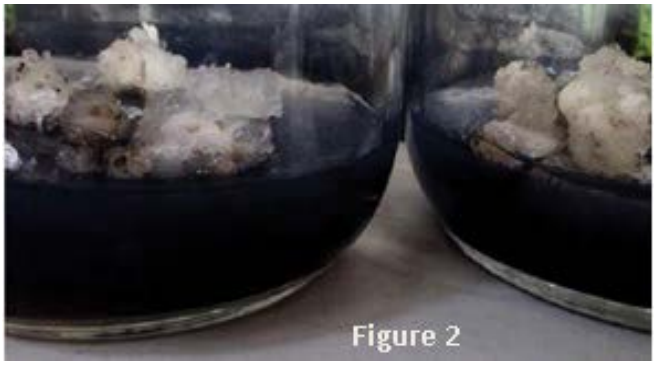

Figure 2: Contamination free and callus promising Leaf sheath explants which sterilized with 1% *NaOCl*.

The present study result showed that when shoot tip explants sterilized with less concentration (1%) of *NaOCl* culture contamination rate was increased. In harmony with the current study, [23] reported that enset shoot tip explants disinfecting by (1.5%) of *NaOCl* for 15 min caused high percentage of contamination. Previously 40% of gion berekina and 2% *NaOCl* were used for enset shoot tip explants sterilization [11,16]; however culture contamination rate was high as compared to the present study. The improvement of explants sterilization in the current study as compared to Genene and Firew, Almaz et al. [11,16] may be attributed to explants source, explants size, time of exposure to the disinfectant or using of Cefotaxime to reduce endophytic contaminants. Similar previous findings showed that explants contamination rate depends on several plant and environmental related factors such as species, age, explant source or size, level of aseptic work, prevailing weather condition and time of exposure to the disinfectants [24]. Moreover, Sodium hypochlorite produced the highest reduction in bacterial and fungal contamination at time intervals between 20-45 min [13].

Sterilization of leaf sheath Explants

ANOVA showed that concentration of *NaOCl* and its interaction with cultivars had highly significant difference ($P<0.001$) in overcoming contamination and improving survival of leaf sheath explants (Table 2).

Table 2: Effect of *NaOCl* concentration on leaf sheath explants sterilization

Cultivars	NaOCl (% v/v)	Mean No of alive and contamination free explants/treatment	Percentage of contamination free and alive explants (%)(mean ± SD)
Mesena	0	0	0 ± 0.0
	0.5	**4.7**	**93.3 ± 11.5**
	1	4	80 ± 0.0
	2	2.6	52 ± 11.5
Yanbule	0	0	0 ± 0.0
	0.5	3	60 ± 0.0
	1	**5**	**100 ± 0.0**
	2	3.7	73.3 ± 11.5
Endale	0	0.7	13.3 ± 11.5
	0.5	**4.7**	**93.3 ± 11.5**
	1	3	60 ± 0.0
	2	1.7	34 ± 0.0
CV			14.8
ISL			***

CV=Coefficient of Variation, ISL=Interaction Significant Level, ***=highly significant and SD=Standard Deviation. In a column, means followed by the same letter are not significantly different at the 5% level by DMRT. Each treatment had four replications with four piece leaf sheath per replication.

In this study, *NaOCl* was notably resulted the highest percentage of aseptic healthy leaf sheath culture as compared to the control (Table 2). All leaf sheath explants of Mesena and Yanbul sterilized with control became contaminated; but for the case of cultivar Endale explants sterilized with control treatment produced 13.3 ± 11.5% contamination free and alive explants. However, clear significance difference between *NaOCl* concentrations was observed with regard to healthy culture establishment. As per results the highest percentage (100%) of alive and contamination free leaf sheath explants were recorded on cultivar Yanbule when explants were sterilized with 1% *NaOCl*, which was found more effective for sterilization and further *in-vitro* response of enset leaf sheath explants (Figure 2). In line with the present study, Satish et al. also reported similar results [25].

Whereas in the case of Mesena and Endale the highest percentage (93.3 ± 11.5%) of survival and contamination free explants were observed at 0.5% *NaOCl*. However, in the case of Mesena, 0.5% *NaOCl* did not give statistically significant difference compared to 1% *NaOCl* for contamination free and surviving explants. It was observed that leaf sheath explants of Endale exposed to elevated concentration (>0.5%) of *NaOCl* showed immediate browning and death. However, cultivar Yanbule was tolerant to *NaOCl* toxicity up to 1%. This is suggesting that varietal difference evokes varying resistance response to the same sterilizing agent. In consistent with the present study previous researchers report showed that, optimum concentration of sterilizing agent must be determined empirically for genotypes to prevent phyto toxicity [15]. Moreover the present study revealed that the tested cultivars showed differential responses in terms of culture contamination rate. Similarly Mulugeta and Van Staden [17] reported that levels of culture contamination were dependant on enset genotype. These contamination differences between genotype may be caused due to presence of genotype dependent endophytic and surface contaminants on the explants.

The use of enset leaf sheath as explant for culture initiation has not been reported elsewhere. The present study showed that, use of leaf sheath was possible to initiate callus (data not shown here) (Figure 2). In line with present study, Novak et al. [26] reported embryogenic callus formation by inoculating leaf sheath explants of triploid cooking banana. Similarly, shoot directly regenerated from leaf explants of silk banana [27]. Therefore, the present leaf sheath explants sterilization study was initiated as baseline for *in-vitro* regeneration and future genetic improvement of different genotype of enset. In addition to surface contaminants, endophytic contaminants were the main challenge during micro propagation work of enset [4]. Therefore, in the present study to prevent contamination which caused by endophytic contaminant antibiotic cefotaxime (500 mg/L) was added in the culture medium.

CONCLUSION

Based on the current result, it is possible to conclude that, effective enset shoot tip and leaf sheath explants sterilization protocol were developed. In general 2% sodium hypochlorite (*NaOCl*) solution was most effective for sterilization

of all tested enset cultivars shoot tip explants to establish sterile cultures. Moreover, 0.5% *NaOCl* concentration was found to be best for leaf sheath explants sterilization of cultivar Mesena and Endale; while for cultivar Yanbul maximum percentage of alive and sterile leaf sheath culture recorded at 1% *NaOCl*. Use of the developed explants sterilization procedure is effective, can save our time and resource and can be followed in the future when enset shoot tip and leaf sheath are used as explants to establish *in-vitro* plantlet propagation or for any these explants related tissue culture application.'

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