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Effect of sterilizing agents on the production of callus in medicinal plant: *Chlorophytum* sps.

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Abstract
Chlorophytum borivilianum Santapau & R.R. Fern and *Chlorophytum tuberosum* (Roxb.) Baker has been considered as critically endangered plants belongs to family Anthericaceae. Different sterilants with different combinations of Dettol, antioxidant solution (ascorbic acid+ citric acid+ autoclaved distilled water), ethanol, hydrogen peroxide, and sodium hypochlorite were used. Among them leaves treated with hydrogen peroxide showed significant growth and survival rate as compared to sodium hypochlorite and antioxidant solutions and dettol treated leaves. The most effective protocol for sterilization tested was washed with distilled water followed by 3% hydrogen peroxide for 5minutes and then rinsed with ethanol. For callus production, leaves were taken as explant and grown under full strength Murashige and Skoog media+ cytokinin (Benzyl adenine-BA) either alone or with 2,4-Dichlorophenoxy acetic acid (2,4-D) at different concentrations on callus. The best results shown was BA 0.7 µm+2, 4 D 0.7µm with 100% callus production. This protocol could be used for secondary metabolite production along with micropropagation of these endangered varieties.

Keywords: Sterilants, Hydrogen peroxide, Sodium hypochlorite, Chlorophytum, Callus

1. Introduction
The inclination towards natural products is amassed progressively. The usage of conventional medicines and medicinal plants in most developing countries as a common basis for keeping good health has been usually witnessed. Further, an increasing dependence on the use of medicinal plants in the industrialized societies has been related to the improvement of several chemotherapeutics from plant species as well as from conventionally used rural herbal preparations. Herbal therapies have attained much more acceptance in the treatment of minor ailments, due to increasing alertness of personal health maintenance through natural products. Undeniably, the market and public plea have been so great that there is a massive extinction threat to many medicinal plants and apparently the loss of genetic diversity.
Chlorophytum borivilianum Santapau & R.R.Fern-CB (critically endangered [1]and *Chlorophytum tuberosum* (Roxb.) Baker –CT (least concern [2]) belongs to family Anthericaceae were important medicinal plants in India and mostly cultivated for its medicinal values. CB is basically recognized by Divya Aushad or White Gold by Indian medicinal system. The species has significant market appeal owing to its marketable use as a remedy and the level of exploitation is very high. The current scenario at national and international level trade is the root of Safed Musli. Due to overexploitation of its roots and tubers, it has been uprooted from its wild sites. This has a severe consequence on its natural restoration. Thus, the inhabitants of this species are diminishing rapidly in the natural habitat[3,4]. Immediate attention should take from the worsening condition of the species in their natural habitat [5]. The improper use and collection of these population and low density of the plant specify that the species possibly will become extinct quickly if proper measures are not taken. Due to its aphrodisiac and rejuvenating properties along with ease in sexual disorders, it has been put in Ayurved as "Vajikaran Rasayana". It is largely used as folkloric medication by indigenous communities of India [3]. The root is used in the traditional method of medicine.
In vivo grown plants were contaminated with microorganisms. This adversely affects the plants grown under *in vitro*. These microbes can hamper the growth of the explant by competing for nutrients, inhibits the culture leads to necrosis, reduced shoot proliferation, rooting and mortality of explant [6]. These contaminants might appear at a later stage of culture which are difficult to eliminate[7]. Frequently sterilants like mercuric chloride, calcium hypochlorite, chlorine gas, ethanol, and sodium hypochlorite, were used as a surface sterilizer for plant and seed materials of various species [8–10].Inappropriately these chemicals unsuccessfully remove contaminants. Mercuric chloride, one of the most unsafe sterilizing agent does require safe handling during the disinfection procedure, while the resulting harmful remains must be disposed of properly. This study tries to emphasize upon the different combinations of sterilization agents including dettol, antioxidant solution (1gm ascorbic acid+5gm citric acid+200ml sterilized distilled water), ethanol, hydrogen peroxide, and sodium hypochlorite. This provides better contaminant proof environment for the developing callus from the leaf as explant.

2. Materials and methods
2.1 Plant material
The explants of both the plants *Chlorophytum borivilianum* Santapau & R.R.Fern and *Chlorophytum tuberosum* (Roxb.) Baker (identified by Dr. M. A. Patel, Research Scientist) were obtained from Medicinal and Aromatic Research Centre, Anand Agriculture University, Anand, Gujarat, India.

2.2 Sterilizing agents treatment and inoculation of explant
For setting up of disinfected free cultures, young and fresh

leaves were taken. These leaves were put under running tap water for 5 minutes and dipped in autoclaved distilled water for 2 minutes followed by H_2O_2 treatment for 2 minutes. The washed leaves were again put in ethanol before cutting into 2-4mm size. In another treatment, bavistin 2% and sodium hypochlorite and lastly washed with autoclaved distilled water before inoculation of leaf cuttings. In another case, antioxidant solutions (ascorbic acid+ citric acid+ autoclaved distilled water) were used instead of these bleach.

For the callus production, the above-treated leaves fragments were grown under growth regulators BA (Benzyl Adenine as cytokinin) either alone (1-2.5 µm) or with 2, 4-D (2, 4-Dichlorophenoxy acetic acid) (0.1-1.5µm BA + 0.1-1.5µm 2, 4-D) with the full strength of Murashige and Skoog media.

3. Results and discussion

3.1 Effect of sterilizing agents

The present study deals with three key surface sterilization methods based on the use of sodium hypochlorite or hydrogen peroxide using leaves as explant with different grades of contamination. Nevertheless, in this study, mercuric chloride was not used as earlier used for surface sterilization of seeds and plant material of many plant species[10–12]. Mercuric chloride is very hazardous because of its high toxicity and more difficult to dispose of a hazardous waste [13,14]. In *Chlorophytum sps*. a lot of contamination problem under *in vitro* conditions appears as it might be a grown under natural habitat with high humidity along with endogenous hormonal and physiological conditions.

Table 1: Effect of different sterilization treatments with different times on *Chlorophytum borivilianum* Santapau & R.R.Fern *and Chlorophytum tuberosum* (Roxb.) Baker (young leaves). -All plant parts washed at the first with an antioxidant solution (1g ascorbic acid+5g citric acid dissolved in 200 ml sterilized distilled water) under aseptically sterilized conditions.-Dettol treatments added as 2ml dettol in 200 ml sterilized distilled water for 10 seconds. CL= Clorox (Sodium hypochlorite).

Time	2 mint			5 mint			10 mint		
Treatment	**%of survival**	**%of cont.**	**%of Death**	**%of survival**	**%of cont.**	**%of Death**	**%of Survival**	**%of cont.**	**%of Death**
5%CL	42.25	57.50	0	41.95	56.90	1.15	39.35	38.85	21.80
10%CL	39.41	42.94	17.65	38.40	40.25	21.35	25.85	18.75	55.40
15%CL	33.33	38.33	28.34	32.28	34.35	33.37	7.35	-	92.65
Dettol+5%CL	54.28	44.25	1.47	54.25	42.35	5.40	34.22	32.85	32.93
Dettol +10%CL	51.42	40.92	7.66	50.35	41.30	8.35	28.75	18.85	52.40
Detttol+15%CL	45.42	32.35	22.40	48.25	30.28	21.47	25.80	17.55	56.65
Detttol+20%CL	44.60	30.45	24.95	49.80	28.45	21.75	20.70	16.87	62.43
1% H_2O_2	40.21	21.22	29.4	38.32	23.25	52.1	12.1	15.2	75.7
2% H_2O_2	48.25	25.35	26.40	43.25	17.35	59.40	40.12	18.30	68.70
3% H_2O_2	**69.12**	**15.11**	**20.11**	61.18	17.1	27.4	54.12	15.1	35.11
F-value	0.816		1.225	0.204		4.89	0.424		2.35
CV	21.1%		61.5%	18.7%		75%	48%		38%
SEM	3.134		3.469	2.71		6.01	4.38		6.73

The effect of various sterilizing agents showed initially no symptoms. As the days progresses it started showing contamination. After 2, 5 and 10 minutes of incubation, the percentage of survival tests, the percentage of contamination and percentage of death were measured. Among the sterilizing chemicals though each one provides survival as well contamination free up to certain extent, most effective was 3% H_2O_2 (Table 1). The latter showed 69.2% survival with 15.11% contamination and 20.11% death rate. Though CL individually 42.25% survival with 0% death percentage but 57.5% contamination which was not suitable for *In vitro* callus production. Concomitantly Dettol with 5% CL provided 54.28% survival, but again the contamination percentage was high (44.25%) which might not be suitable. Therefore, in this study, the most effective protocol for sterilization tested was washed with distilled water followed by 3% hydrogen peroxide for 5minutes and then rinsed with ethanol.

3.2 *In vitro* callus production

The callus production started after 15 days of incubation with a photoperiod of 16hr light and 8hrs dark.

Table 2: Effect of hormonal combination cytokinin BA either alone or with 2, 4-D at different concentrations on callus differentiation from leaf explant. Each value represents the mean ± SE of three replicate experiments with *Chlorophytum borivilianum* Santapau & R. R. Fern-CB and *Chlorophytum tuberosum* (Roxb.) Baker-CT leaf per treatment in each experiment. MS medium was used with all the growth regulators. CV-Coefficient of variation; SEM- standard error of mean; F value

Treatments	Percent cultures showing callus (CB)	Percent cultures showing callus (CT)
I		
BA 1.0µm	0	0
BA 1.5 µm	0	0
BA 2.0µm	0	0
BA 2.5 µm	0	0
II		
BA 0.1 µm+2,4 D 0.1µm	10	0
BA 0.2 µm+2,4 D 0.2µm	11.1	0
BA 0.3 µm+2,4 D 0.3µm	11.5	10.4
BA 0.4 µm+2,4 D 0.4µm	20	18.1
BA 0.5 µm+2,4 D 0.5µm	30	31.1

BA 0.6 µm+2,4 D 0.6µm	66.5	46.5
BA 0.7 µm+2,4 D 0.7µm	100	70.0
BA 0.8 µm+2,4 D 0.8µm	80	78.1
BA 0.9 µm+2,4 D 0.9µm	60.2	100
BA 1.0 µm+2,4 D 1.0µm	20	50.1
BA 1.1 µm+2,4 D 1.1µm	20	20.4
BA 1.2 µm+2,4 D 1.2µm	0	0
BA 1.3 µm+2,4 D 1.3µm	0	0
BA 1.4 µm+2,4 D 1.4µm	0	0
BA 1.5 µm+2,4 D 1.5µm	0	0
F value	1.16	0.857
CV	74%	64%
SEM	10.98	10.17

Chlorophytum borivilianum Santapau & R. R. Fern-CB showed better results at BA 0.7 µm+2, 4 D 0.7µm concentration where 100percent callus production as compared to BA 0.8 µm+2, 4 D 0.8µm in *Chlorophytum tuberosum* (Roxb.) Baker-CT (Table 2) (Plate 1). This might be due to more endogenous hormonal content in CB as compared to CT. The various day's interval callus produced could be used for further secondary metabolite production. Literature gave emphasis on a combination of 70% ethanol, tween 20 and distilled water as in *Wattakaka volubilis* [15]; teepol with fungicide bavistin (2%) and antibiotic gentamycin sulphate (0.01%) treated in *Cyrtanthus mackenii* [16].

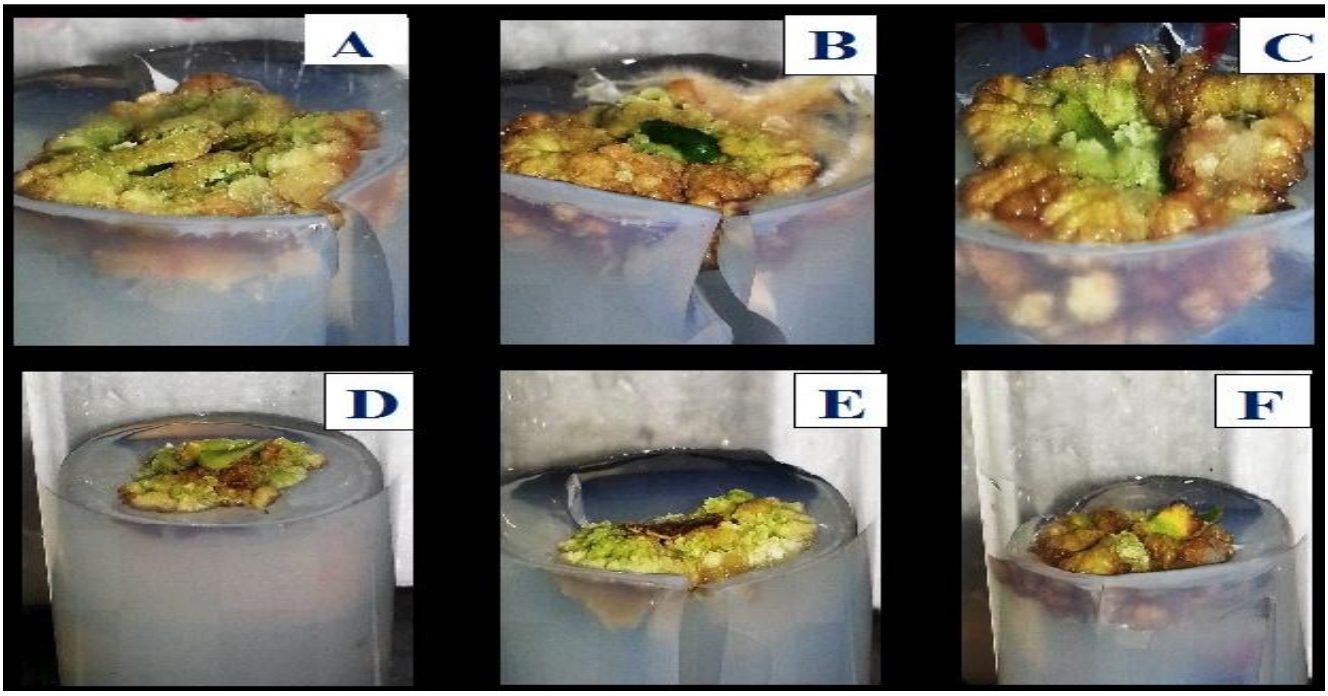

Fig 1: Callus production (A-C) in *Chlorophytum borivilianum* Santapau & R.R.Fern-CB and (D-F) in *Chlorophytum tuberosum* (Roxb.) Baker-CT.

4. Conclusion

Our findings suggest that hydrogen peroxide is the best among the sterilization treatments, with the lowest contamination and highest survival rates. 3% H_2O_2 for 2minutes was the best sterilizing treatments. This study provides an efficient protocol for both sterilizing treatments and establishment of callus production in *Chlorophytum species*.

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