

Original Research Article

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Surface Sterilization Protocol for Nodal and Inter Node Explants of Kankoda (*Momordica dioica* Roxb.)

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ABSTRACT

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To standardize the sterilization procedure for nodal segment and internodes explants of female Kankoda. Comparison was done between two important sterilizing agents, mercuric chloride (0.1%) and sodium hypochlorite (1%) with four time duration 3, 5, 7 and 10 minutes and in combinations. Result showed that amongst the two sterilants i.e. HgCl₂ and NaOCl in combination was found better for controlling the infection. Mercuric chloride (HgCl₂) for 5 minute and sodium hypochlorite (NaOCl) for 5 minute in combination was selected for suitable sterilization agents after 10 minute of DDW wash, 10 minute dip in bavistin and at last washed with double distilled water for nodal segments (83.33 % survival) and internodes segments (83.33 % survival) explants of Kankoda. This is the first report of successful sterilization and reduced contamination of explants from naturally field grown Kankoda by using HgCl₂ and NaOCl sterilizing agents in combination for standardizing efficient *in vitro* surface sterilization protocol.

Introduction

Spine gourd or Teasle gourd or Kankoda belongs to the family Cucurbitaceae and under the genus *Momordica*. *Momordica* genus (annual or perennial climbers) contains about 80 species (Raj *et al.*, 1993). This genus is native of tropical regions of Asia with extensive distribution in China, South East Asia, Japan, Polynesia besides tropical Africa and South America. As many of the species of this genus have been found to grow wildy in India, Bangladesh, Sri Lanka, Myanmar, Malaysia, etc. (Hooker, 1879) it indicated that this region might be the origin of *Momordica*

dioica. The genus *Momordica* possesses different sex forms (monoecious, dioecious, hermaphrodite) and x=14 basic chromosome number. Kankoda, *M. dioica* Roxb. (2n = 2x = 28) and its wild relative, *M. cochinchinensis* Spreng. (2n = 4x = 56) are dioecious and mainly propagate vegetatively through tuberous root (Rashid, 1993). Both the species are perennial, tuberous rooted but differ in their stem, leaf, fruit and seed characters.

It grows in warm and humid weather and tuberous roots are planted in pits. It is an important vegetable in the Indian subcontinent and Bangladesh. It has many

advantages, like high market price, good nutritional value and longer keeping quality. The tender green fruits of Kankoda are used for culinary purpose. Sometimes fruits are sliced, blanched and dried for further use. In some parts, the young tender leaves are used as vegetable. Besides being a vegetable, the seeds of Kankoda yield oil which can be used as an illuminant and also as an admixture with drying oils in formulation of paints and varnishes.

The plant was reported to exhibit anti-diabetic, anti-cancer, anti-fertility abortifacient, anti-inflammatory, antioxidant activity, cure jaundice and bleeding pile properties (Bawara *et al.*, 2010). Owing to its multifold uses, its systematic cultivation would be a boon both for horticulture as well as pharmaceutical industry especially in tropical countries like India. Fruits contain high amounts of protein, calcium, phosphorous, iron, and highest amount of carotene amongst the cucurbitaceous vegetables (Bharathi *et al.*, 2007).

It is mainly propagated by tubers. Propagation by seeds and cuttings is not very common. However, tuberous roots have got dormancy and they sprout only at onset of monsoon. Moreover, the multiplication rate of tubers is very low. Due to lack of technical know-how, the success of rooting of cuttings is very low. Hard and impermeable seed coat possesses problem in germination of seeds in Kankoda. Similarly, sex prediction of seed propagated plant is very difficult before flowering because of dioecious and highly cross pollinated nature.

So far not much systematic efforts have been made to overcome the above problems and crop remained underutilized/neglected from research point of view. Systematic approach can result into some solutions to overcome these problems. Also the natural population of

the plant is gradually decreasing due to habitat destruction and hence, conservation of this crop biodiversity is necessary.

The improvement of *M. dioica* through conventional breeding techniques has several limitations hence for successful application of plant tissue culture for large scale propagation of Kankoda, which requires an efficient explants sterilization system. Therefore, the present investigation was aimed with to develop an efficient explants sterilization protocol in Kankoda.

Materials and Methods

The Kankoda female genotype was collected from AICRN on Potential Crops and present study was carried out at State Level Biotechnology Centre, Department of Agricultural Botany, MPKV, Rahuri during *kharif* 2014-15. This single genotype of Kankoda was taken for regeneration. The two explants namely, nodal segments and internodes explants of female genotype were used for this experiment. These explants were collected from 45 days old plants after sprouting in the field.

Chemicals

Various chemicals of Qualigens Laboratory Pvt. Ltd were used for media preparation and sterilization of explants. Organic supplements like myoinositol were also used.

Cleaning of glassware's

It is important to use cleaned glassware for growth of tissue culture *in vitro*. This was achieved by boiling all glassware's in liquid soap solution for 1 hour, followed by thoroughly rinsing with tap water and immersing in nitric acid (30%) for 2 hours and then removing traces of acid by thoroughly washing with tap water. Then

glassware's were rinsed with double distilled water and they were allowed to dry overnight at room temperature.

Sterilization of media, glassware and instruments

The object of sterilization was to make media, glassware and instruments free from microorganisms. For this, glassware such as conical flasks, test tubes, glass bottle etc., were plugged with non-absorbent cotton. Then petri dishes were wrapped with wrapping paper (aluminum foil). Forceps and scalpels were covered with brown paper. Afterwards the mouth ends of graduated pipettes were plugged with non-absorbent cotton. Individually they were wrapped with wrapping paper. All glassware and instruments were then autoclaved at 121°C and 15 lbs pressure for 20 minutes. Finally they were kept in hot air oven for drying.

Preparation of stock solutions

It was found convenient to prepare the concentrated stock solutions of macro and micro nutrients, vitamins and plant growth regulators. The media used was Murashige and Skoog's (MS media).

Surface sterilization

Sterilization procedure was carried out in three steps. In primary sterilization, all explants were washed with double distilled water for 10 minutes. In secondary sterilization, all the explants were treated with 0.1% bavistin concentration for 10 minutes and washed with double distilled water for 10 minutes. In tertiary sterilization, Mercuric chloride (0.1%) and Sodium hypochlorite (1%) were used for study with treatment durations of 3, 5, 7 and 10 minutes with all possible combinations and alone were used and all explants were washed with double

distilled water for 10 minutes after every treatment for complete removing of traces of chemicals.

To evaluate the response of different chemicals, implantations of sterilized explants were done using without hormones MS medium. The cultures were placed in culture growth room. The cultures were kept in a growth chamber for one month, at 25°C, with 16 hours photoperiod and 3500 lx of light intensity. The observations were recorded up to 30 days. Twelve explants were used in each sterilization treatment, and each treatment was replicated thrice. The observations were recorded in percentage. The effect of surface sterilizing treatments *i. e.* infection per cent, dead per cent and survival per cent of cultures.

Cultural conditions

All the cultural operations including sterilization, inoculation and transfer were carried out in a horizontal laminar air flow chamber using sterilized instruments. Before inoculation, the chamber was smeared with 70% ethyl alcohol and exposed to UV light for 15-20 minutes. The cultured tubes/bottles after inoculation were incubated under dark at 25±1° C.

Data analysis

All the studies were done in laboratory under well defined conditions of the medium, growth, temperature and light. Completely Randomized Design (CRD) was employed for the experiment and the data were analyzed in SAS (Statistical analysis system).

Results and Discussion

In primary sterilization, all explants were washed with double distilled water for 10 minutes. In secondary sterilization, all the

explants were treated with 0.1% bavistin concentration for 10 minutes and washed with double distilled water for 10 minutes. In tertiary sterilization, mercuric chloride (0.1%) and sodium hypochlorite (1%) were used for

study with duration of 3, 5, 7 and 10 minutes with all possible combinations and alone and all explants were washed with double distilled water for 10 minutes for removal of chemical traces (Fig. 1 and 2; Table 1 and 2).

Table.1 Percentage of contamination, percentage of non grown culture and percentage of survival in nodal segment explants after sterilization using various concentrations of sterilizing agents for different time duration

SN	Treatments	Treatment details	Time (min.)	Contamination in nodal explants (%)	Non-grown cultures or % death in nodal explants	Survival % in inter nodal explants
1	S1	Control (DDW washing)	10	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
2	S2	DDW + Bavistin 0.1%	10+10	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
3	S3	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+3	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
4	S4	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+5	91.68 (73.23)	0.00 (0.00)	8.33 (16.77)
5	S5	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+7	66.65 (54.73)	0.00 (0.00)	33.35 (35.27)
6	S6	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+10	0.00 (0.00)	33.33 (35.26)	66.67 (54.74)
7	S7	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+3	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
8	S8	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+5	66.65 (54.73)	0.00 (0.00)	33.35 (35.27)
9	S9	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+7	5.55 (11.18)	19.45 (26.07)	75.00 (60.00)
10	S10	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+10	0.00 (0.00)	66.68 (54.74)	33.33 (35.26)
11	S11	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+3	66.65 (54.73)	0.00 (0.00)	33.35 (35.27)
12	S12	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+5	50.00 (45.00)	0.00 (0.00)	50.00 (45.00)
13	S13	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+7	0.00 (0.00)	33.33 (35.26)	66.68 (54.74)
14	S14	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+10	0.00 (0.00)	75.00 (60.00)	25.00 (30.00)
15	S15	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+5+3	66.65 (54.73)	0.00 (0.00)	33.35 (35.27)
16	S16	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+5+5	0.00 (0.00)	16.67 (24.10)	83.33 (65.90)
17	S17	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+5+7	0.00 (0.00)	50.00 (45.00)	50.00 (45.00)
18	S18	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+5+10	0.00 (0.00)	91.68 (73.23)	8.33 (16.77)
19	S19	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+3	16.65 (24.08)	41.68 (40.21)	41.67 (40.20)
20	S20	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+5	0.00 (0.00)	72.23 (58.25)	27.78 (31.75)

21	S21	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+7	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
22	S22	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+10	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
23	S23	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+3	0.00 (0.00)	83.34 (65.91)	16.66 (24.09)
24	S24	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+5	0.00 (0.00)	91.68 (73.23)	8.33 (16.77)
25	S25	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+7	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
26	S26	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+10	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
			Minimum	0.00	0.00	0.00
			Maximum	100.00 (90.00)	100.00	83.33
			Mean	31.94 (28.17)	36.59	26.08
			SE±	0.54 (1.10)	0.53	0.34
			CD at 5%	1.54 (3.11)	1.50	0.98
			CV%	2.95 (6.74)	2.49	2.28

Table.2 Percentage of contamination, percentage of non grown culture and percentage of survival in inter-node segment explants after sterilization using various concentrations of sterilizing agents for different time duration

Experiments	Treatment details	Time (min.)	Contamination in inter node explants (%)	Non-grown cultures or % death in internode explants	Survival % in inter node explants
S1	Control (DDW washing)	10	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
S2	DDW + Bavistin 0.1%	10+10	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
S3	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+3	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
S4	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+5	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
S5	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+7	75.00 (60.00)	0.00 (0.00)	25.00 (30.00)
S6	DDW + Bavistin 0.1% + HgCl ₂ 0.1%	10+10+10	25.00 (30.00)	8.33 (16.77)	66.67 (54.74)
S7	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+3	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
S8	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+5	100.00 (90.00)	0.00 (0.00)	0.00 (0.00)
S9	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+7	58.33 (49.79)	0.00 (0.00)	41.67 (40.21)
S10	DDW + Bavistin 0.1% + NaOCl 1.0%	10+10+10	0.00 (0.00)	66.68 (54.75)	33.33 (35.26)
S11	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+3	91.68 (73.23)	0.00 (0.00)	8.33 (16.77)
S12	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+5	75.00 (60.00)	0.00 (0.00)	25.00 (30.00)
S13	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+7	16.68 (24.08)	8.33 (16.77)	75.00 (60.00)
S14	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+3+10	0.00 (0.00)	66.68 (54.74)	33.33 (35.26)
S15	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+5+3	66.65 (54.73)	0.00 (0.00)	33.35 (35.27)
S16	DDW + Bavistin 0.1% + HgCl₂ 0.1 % + NaOCl 1.0%	10+10+5+5	5.56 (11.18)	11.13 (19.23)	83.33 (65.90)
S17	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+5+7	0.00 (0.00)	50.00 (45.00)	50.00 (45.00)
S18	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+5+10	0.00 (0.00)	91.68 (73.23)	8.33 (16.77)
S19	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+3	16.65 (24.08)	41.68 (40.21)	41.67 (40.20)
S20	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+5	0.00 (0.00)	75.00 (60.00)	25.00 (30.00)

21	S21	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+7	0.00 (0.00)	94.45 (78.82)	5.55 (11.18)
22	S22	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+7+10	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
23	S23	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+3	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
24	S24	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+5	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
25	S25	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+7	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
26	S26	DDW + Bavistin 0.1% + HgCl ₂ 0.1 % + NaOCl 1.0%	10+10+10+10	0.00 (0.00)	100.00 (90.00)	0.00 (0.00)
			Minimum	0.00	0.00	0.00
			Maximum	100.00 (90.00)	100.00	83.33
			Mean	39.64 (35.66)	34.98	21.02
			SE±	0.54 (1.10)	1.20	1.10
			CD at 5%	1.54 (3.11)	3.42	3.11
			CV%	2.38 (5.33)	5.96	9.03

Fig.1 Promising treatment combination for % contamination, % dead explants and % survival in nodal explants after sterilization with various concentrations of sterilizing agents for different time duration

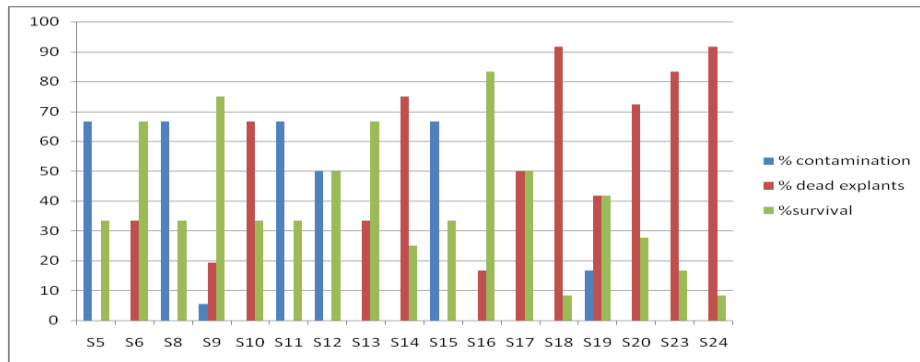


Fig.2 Promising treatment combination for % contamination, % dead explants and % survival in inter-node explants after sterilization with various concentrations of sterilizing agents for different time duration

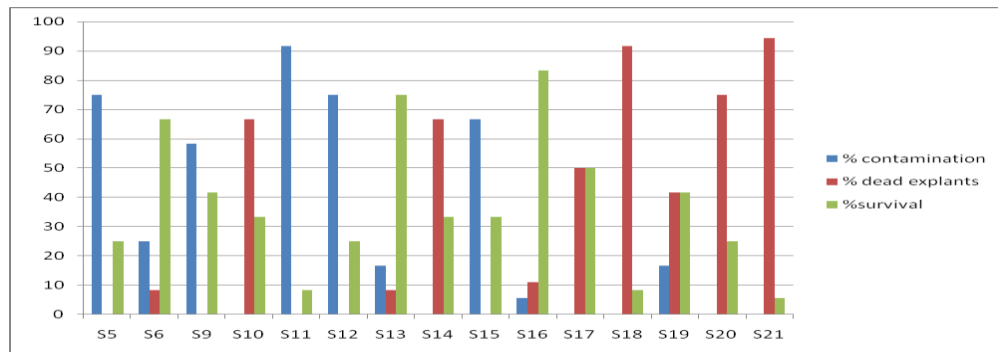


Plate.1 Standardizing efficient *in vitro* surface sterilization protocol through DDW + Bavistin 0.1% + HgCl₂ 0.1% + NaOCl 1.0% (10+10+5+5) for inter node explant





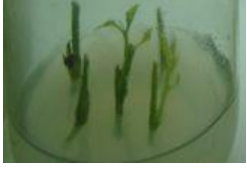



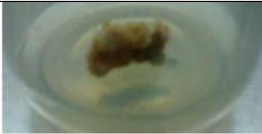

		
Non-grown cultures in inter-node explants		Survival of inter-node explants
		
	Survival of inter-node explants	
		
Survival of inter-node explants		Survival of inter-node explants

Plate.2 Standardizing efficient *in vitro* surface sterilization protocol through DDW + Bavistin 0.1% + HgCl₂ 0.1% + NaOCl 1.0% (10+10+5+5) for nodal explant

		
Non-grown cultures in nodal explants		Survival of nodal explants
		
	Non-grown cultures in nodal explants	
		
Non-grown cultures in nodal explants		Survival of nodal explants

To evaluate the response of different treatment combinations, implantations of sterilized explants were done using without hormones MS medium and results showed that with increase of time the infection was decreases in both the sterilizing agents for all the two explants when sterilized explants

individually or in combination of both sterilizing agents.

When HgCl₂ 0.1% was used alone, nodal segments and internodes explants recorded highest percentage (66.67%) in treatment DDW + Bavistin 0.1% + HgCl₂ 0.1%

(10+10+10 minutes), HgCl₂ 0.1 % has been previously used for sterilization of different explants in different crops. Thakur *et al.*, (2011) surface sterilized axillary buds of *Momordica balsamina* with 0.1% mercuric chloride solution for 7 minutes and Mustafa *et al.*, (2013) surface sterilized explants of Kankoda internodes and nodes explants with 0.1% HgCl₂ for 7-8 minutes.

When NaOCl 1.0 % alone was used, the highest percentage of survival of cultures was recorded in treatment DDW + Bavistin 0.1% + NaOCl 1.0 % (10+10+7 minutes) with 75.00 % and 41.67 % by nodal segment explants and internode explants respectively. The NaOCl 1.0 % has been extensively for sterilization of different explants with different time duration in different crops. Badoni and Chauhan (2010) developed *in vitro* sterilization protocol for micropropagation of potato cv. 'Kufri Himalini' by using sterilizing agent 1.0 % NaOCl with 8 minutes time duration for explants sterilization.

For nodal segment explants, the survival rates ranged from 0.0 to 83.33%. The treatment DDW + Bavistin 0.1% + HgCl₂ 0.1 % + NaOCl 1.0% (10+10+5+5) recorded maximum survival percentage (83.33 %) with 0.0 % contamination and 16.67 % non-growing culture for nodal segment explants. For internode explants, the survival of cultures ranged from 0.0 to 83.33%. The treatment DDW + Bavistin 0.1% + HgCl₂ 0.1 % + NaOCl 1.0% (10+10+5+5) recorded maximum survival percentage (83.33 %) with 5.56 % contamination and 11.33 % non-growing culture for internodes explants.

In treatments involving combination of HgCl₂ and NaOCl, the highest percentage of survival of cultures were recorded in treatment i.e. DDW + Bavistin 0.1% + HgCl₂ 0.1 % + NaOCl 1.0 % (10+10+5+5 minutes)

with 83.33 % for nodal segment explants as well as internode explants. The combinations of two different sterilizing agents for surface sterilization have been used in various reports. Miche and Balandreau (2001) analyzed the effect of combination of hydrogen peroxide and hypochlorite was used to surface sterilize rice seeds. Colgecen *et al.*, (2011) analyzed the effects of 1.25 % sodium hypochlorite and Plant Preservative Mixture (PPM) on surface sterilization in *A. densiflora*

Suitable surface sterilization agents

While comparing the effect of HgCl₂ and NaOCl alone and in all possible combination with varying times, the treatment Bavistin 0.1% + HgCl₂ 0.1 % + NaOCl 1.0% (10+10+5+5 minutes time respectively) was always found better than any other treatments for suitable sterilization combination for both the explants used in this investigation.

This is the first report of successful sterilization and reduced contamination of explants from naturally field grown Kankoda (*Momordica dioica* Roxb., by using HgCl₂ and NaOCl sterilizing agents in combination for standardizing efficient *in vitro* surface sterilization protocol. These results were found better for controlling the infection and it had not any adverse effect on explants even in long duration.

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