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Optimizing sterilization protocols for *in vitro* culture establishment of finger lime (*Citrus australasica* F. Muell): A comprehensive investigation

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Abstract
Finger lime (*Citrus australasica F. Muell*) stands out among Australian citrus species, gaining attention for its unique finger-like fruits and the popular term "lime caviar" attributed to its juice vesicles. The rise in demand for finger lime has intensified the need for efficient tissue culture methods to propagate selected genotypes, free from contamination. This research focuses on establishing a robust surface sterilization protocol for *in vitro* culture initiation of finger lime. The study explores the impact of various exposure times of sodium hypochlorite (4%) on different explant types (leaf, internode, nodal segment, and shoot tip). Results indicate that a 10-minute exposure to sodium hypochlorite (NaOCl) (4%) is optimal for minimizing contamination and maximizing survival. Furthermore, the investigation extends to the combined effect of mercuric chloride ($HgCl_2$) with sodium hypochlorite (4%). To enhance the sterilization process, ethanol (70%) exposure times are introduced in combination with the best sodium hypochlorite and mercuric chloride treatments. The findings reveal that a 45-second ethanol treatment complements the sterilization process, achieving low contamination levels in nodal and shoot tip explants. However, a trade-off is observed with a decrease in survival rates due to the potentially lethal effects of higher concentrations and exposure times. The study provides valuable insights into the initiation of response, days required for shoot induction, and overall health of the explants. Nodal segments emerge as the most responsive explants, exhibiting 100% initiation of response across various treatments.

Keywords: *In vitro* culture, surface sterilization, sodium hypochlorite, mercuric chloride, ethanol, contamination

Introduction
Finger lime (*Citrus australasica* F. Muell) is one among the five Australian citrus species. It is also known by the names Australian lime and caviar lime. This species was recently reclassified into the *Citrus* genus based on updated taxonomic studies (Mabberley, 1998) [8] after originally being placed in the Microcitrus genus (Swingle, 1915) [11]. In their natural environment, finger limes are little, thorny trees or shrubs that can grow up to 6 metres tall. The fruit has a recognizable finger-like shape, can reach a length of 12 cm, and is frequently slightly curled, narrowing at the tip and base. The pulp can be any coloured from green to yellow to different shades of red, although the peel is often either green or red. Due to the juice vesicles loose adhesion and resemblance to caviar, the pulp is frequently referred to as "lime caviar" (Delort and Yuan, 2018) [5]. Due to more people using the fruit pulp, the demand for the fruit has surged dramatically in recent years. Finger lime cultivars used for commercial production are chosen saplings from eastern Australian forests (New South Wales and Queensland) (Rennie, 2017) [10]. These Australian states also produce the majority of the finger lime fruit that is sold internationally.
In tissue culture, sterilization is a crucial and fundamental component because it enables the *in vitro* propagation of chosen genotype progenies free from external and internal contamination. Explants can be taken from field grown plants or from *in vitro* grown plants. For the establishment of explants *in vitro*, the growth conditions of the stock field-growing plants are crucial. Numerous microorganisms can be seen on the surface of the plant material growing in the field. The explants obtained from field-grown seedlings include several microbial pathogens, including fungi, bacteria, and adherent soil particles; as a result, they must first undergo a comprehensive and efficient surface sterilizing procedure before being cultured.

The development of *in vitro* cultures may be hindered by these microbes, which might be challenging to eradicate. The bacterial and fungal contamination issues can be readily avoided with a routine process for surface-disinfection in citrus if the stock material is obtained during the warm humid season (a period that correlates with a vigorous growth). The entire plant is clipped and defoliated for new shoot growth to encourage aggressive plant growth during slow growth periods (during the dry or cold season) (Carimi and Pasquale, 2003) [2]. There are no reports on sterilization procedure for *in vitro* clean culture establishment in finger lime. Therefore, this study aimed to select the best surface sterilization procedure for leaf, stem tip, internodal and nodal explants of finger lime.

Materials and Methods

The explants for the experiment were collected from mother plant of Finger lime, which was grown in the polyhouse of College of Horticulture, Sirsi. The explants were cut to the required size and then washed. A Few drops of liquid detergent were added, and explants were thoroughly cleaned for 15 to 20 minutes under running tap water. Subsequently, these explants were treated in a fungicide solution of bavistin (0.2%) and citrimide (0.05%), along with a few drops of Tween 20, for about 4 hours. These treated explants were then transferred to a LAF cabinet, where further treatments were carried out. In this study, diverse explants types, such as shoot tip, nodal segment, internodal and leaf segment were cultured on MS medium as the basal medium along with BA (0.5 mg/L) to optimize the efficient surface sterilization process. The treatments included the use of sodium hypochlorite (4%) alone for different time intervals, a combination of the best treatment of sodium hypochlorite (4%) with different concentrations and exposure times of mercuric chloride and a combination of best treatment of sodium hypochlorite (4%) and mercuric chloride with different exposure timings of ethanol (70%) were tried to achieve a minimal contamination, maximum survival, and healthy explants with optimal responses.

The experimental design used for the study was a Completely Randomized Design (CRD). Where values were 0% or 100%, arcsin (1/4 n) and arcsin (100-1/4 n), where n is the number of observations that sum up the percentage, were substituted, accordingly (Zar, 1984). The small whole numbers consisting 0 were converted using square root transformation.

Observations recorded

The observations were recorded every week for upto four weeks and accumulated data of one month is represented in the table. The data were recorded for parameters such as contamination (Bacteria/Fungal) (%), health of explants (+ = least healthy, ++ = moderate healthy and +++ = healthy), initiation of response in explants (%), days taken for shoot induction, and the survival of explants (%).

Results and Discussion

Effect of exposure time of sodium hypochlorite (4%) on explants of finger lime

To optimize the efficient surface sterilization protocol, explants were exposed to sodium hypochlorite (4%) solution for different time intervals, then rinsed with sterile distilled water five times and cultured on MS medium. In the present study, the highest fungal contamination was recorded in the control and as the exposure time to sodium hypochlorite (4%) increased contamination also decreased in leaf and internodal explants (Table 1). Surface sterilization at higher concentrations may be caused by the longer-term phytotoxic effects of local bleach that contains 0.5 percent chlorine (Felek *et al*., 2015) [6]. In this study, the lowest fungal contamination (63.33%) in leaf and in internode (46.67%) was found in sodium hypochlorite (4%) treated for 10 minutes. However in nodal and shoot tip explants 100 percent contamination was noticed during fourth week in all treatments. The bacterial contamination did not notice much in all explants because the growth of fungal contamination was very quick in the culture and it covered all the space. The treatment with sodium hypochlorite alone was not useful in eliminating the contamination, completely. Similarly in pineapple, sterilization with sodium hypochlorite alone resulted in infected explants (Abul-Soad *et al*., 2006) [1].

Table 1: Effect of exposure time of sodium hypochlorite (4%) on contamination (%) and health of explants of finger lime.

Exposure time (min)	Contamination (%)				Health of explants			
	Leaf	Internode	Nodal segment	Shoot tip	Leaf	Internode	Nodal segment	Shoot tip
0	100.00 (89.71)^{a}	100.00 (89.71)^{a}	100.00 (89.71)	100.00 (89.71)	++	+	+	+
3 min	83.33 (66.15)^{b}	100.00 (89.71)^{a}	100.00 (89.71)	100.00 (89.71)	++	+	+	++
4 min	80.00 (63.44)^{bc}	83.33 (65.91)^{b}	100.00 (89.71)	100.00 (89.71)	+++	++	+	++
5 min	73.33 (59.00)^{cd}	63.33 (52.73)^{c}	100.00 (89.71)	100.00 (89.71)	+++	++	++	++
6 min	73.33 (59.00)^{cd}	60.00 (50.77)^{d}	100.00 (89.71)	100.00 (89.71)	+++	+++	++	++
7 min	70.00 (56.79)^{cd}	53.33 (46.91)^{e}	100.00 (89.71)	100.00 (89.71)	+++	+++	++	++
8 min	70.00 (56.79)^{cd}	50.00 (45.00)^{f}	100.00 (89.71)	100.00 (89.71)	+++	+++	++	+++
9 min	66.67 (54.78)^{d}	46.67 (43.09)^{g}	100.00 (89.71)	100.00 (89.71)	+++	+++	++	+++
10 min	63.33 (52.78)^{d}	46.67 (43.09)^{g}	100.00 (89.71)	100.00 (89.71)	+++	+++	++	+++
S.E m±	2.80	0.29	0.00	0.00	-	-	-	-
LSD at 0.01	6.81	1.56	NS	NS	-	-	-	-
CV	4.67	1.56	0.00	0.00	-	-	-	-

In this study, the survival (Table 2) and health of explants (Table 1) were comparatively good in case of sodium hypochlorite (4%) treated for 10 minutes in all explants of finger lime. No initiation in response was noted in leaf explants where as in internodal, nodal and shoot tip explants initiation of response increased with the exposure time to sodium hypochlorite (4%). Nodal segments were highly responsive and 100 percent was found in exposure timings from six to ten minutes. The internodal explants showed callus induction. The direct shoot induction was recorded only in shoot tips and nodal segments and days taken for shoot induction (11 to 15 days) was not much influenced by sodium hypochlorite (4%) treatment for both explants (Table 3). In earlier studies, for shoot tip and nodal segments of seedlings of jackfruit, treatment with sodium hypochlorite (0.75%) for 15 minutes was effective and resulted in highest (46.60%) survival and lowest (33.33%) contamination (Khan *et al*., 2010) [7].

Table 2: Effect of exposure time of sodium hypochlorite (4%) on survival (%) of explants of finger lime

Exposure time (min)	Survival (%)			
	Leaf	Internode	Nodal segment	Shoot tip
0	76.67 (61.12)^{e}	0.00 (0.29)^{e}	0.00 (0.29)^{e}	20.00 (26.57)^{i}
3 min	86.67 (68.63)^{d}	0.00 (0.29)^{e}	0.00 (0.29)^{e}	46.67 (43.09)^{h}
4 min	90.00 (71.57)^{c}	0.00 (0.29)^{e}	3.33 (10.51)^{d}	53.33 (46.91)^{g}
5 min	90.00 (71.57)^{c}	0.00 (0.29)^{e}	3.33 (10.51)^{d}	56.67 (48.84)^{f}
6 min	93.33 (75.04)^{b}	20.00 (26.57)^{d}	3.33 (10.51)^{d}	60.00 (50.77)^{e}
7 min	93.33 (75.04)^{b}	30.00 (33.21)^{c}	3.33 (10.51)^{d}	63.33 (52.73)^{d}
8 min	96.67 (79.48)^{a}	30.00 (33.21)^{c}	16.67 (24.09)^{c}	70.00 (56.79)^{c}
9 min	96.67 (79.48)^{a}	63.33 (52.73)^{b}	33.33 (35.25)^{b}	73.33 (58.91)^{b}
10 min	96.67 (79.54)^{a}	100.00 (89.71)^{a}	56.67 (48.84)^{a}	80.00 (63.44)^{a}
S.E m±	0.23	0.02	0.12	0.18
LSD at 0.01	1.97	0.49	1.40	1.71
CV	1.14	0.80	3.56	1.46

Table 3: Effect of exposure time of sodium hypochlorite (4%) on initiation of response (%) and days taken for shoot induction in explants of finger lime.

Exposure time (min)	Initiation of response (%)			Days taken for shoot induction	
	Internode	Nodal segment	Shoot tip	Nodal segment	Shoot tip
0	0.00 (0.29)^{d}	70.00 (56.79)^{e}	6.67 (14.75)^{e}	0.00 (1.00)^{d}	0.00 (1.00)^{b}
3 min	0.00 (0.29)^{d}	80.00 (63.44)^{c}	13.33 (21.35)^{d}	11.30 (3.51)^{c}	0.00 (1.00)^{b}
4 min	0.00 (0.29)^{d}	83.33 (65.91)^{b}	13.33 (21.35)^{d}	13.33 (3.78)^{abc}	0.00 (1.00)^{b}
5 min	0.00 (0.29)^{d}	76.67 (61.12)^{d}	20.00 (26.57)^{c}	15.00 (4.00)^{a}	0.00 (1.00)^{b}
6 min	30.00 (33.21)^{b}	100.00 (89.71)^{a}	23.33 (28.86)^{c}	15.00 (4.00)^{a}	0.00 (1.00)^{b}
7 min	30.00 (33.21)^{b}	100.00 (89.71)^{a}	30.00 (33.21)^{b}	14.66 (3.96)^{a}	15.00 (4.00)^{a}
8 min	13.33 (21.35)^{c}	100.00 (89.71)^{a}	30.00 (33.21)^{b}	14.33 (3.92)^{ab}	15.52 (4.06)^{a}
9 min	32.00 (34.44)^{b}	100.00 (89.71)^{a}	50.00 (45.00)^{a}	13.00 (3.74)^{abc}	15.41 (4.05)^{a}
10 min	43.33 (41.16)^{a}	100.00 (89.71)^{a}	56.67 (48.84)^{a}	12.33 (3.65)^{bc}	14.32 (3.91)^{a}
S.E m±	0.34	0.03	0.92	0.07	0.03
LSD at 0.01	2.37	0.66	3.89	0.29	0.14
CV	5.52	0.37	5.47	3.42	2.47

The selection of the explant is thought to play a significant role in the induction of shoot regeneration. The tissue's responsiveness can be influenced by both the size and type of the explant. Larger explants tend to possess more significant nutritional reserves and plant growth regulators, which can contribute to the culture's support. No response was observed in leaf explants and response in the form of callus was recorded in internodal explants, only in shoot tips and nodal segments direct shoot regeneration was observed so they were considered as best explants for regeneration in finger lime and selected for further study.

Effect of different concentrations of mercuric chloride combined with best exposure timings of sodium hypochlorite (4%) on explants of finger lime

In the present study, the best exposure timings of eight, nine and ten minutes were selected based on the above findings as they comparatively inhibited contamination than other treatments and they are combined with different concentration and exposure timings of mercuric chloride to get the best results in finger lime.

In this study, contamination was low in both nodal (43.33%) and shoot tip (30%) explants when treated with combination sodium hypochlorite (4%) for ten minutes + mercuric chloride (0.1%) for eight minutes (Table 4). But the survival and the health of explants were not good in that treatment. This may be due to increased concentration and exposure timings of sterilizing agents. Higher concentrations and exposure time could have a phytotoxic effect (Felek *et al*., 2015) [6]. In the present study, the higher survival (100%) (Table 5) and healthy explants (Table 4) were obtained in sodium hypochlorite (4%) for ten minutes + mercuric chloride (0.05%) for eight minutes, sodium hypochlorite (4%) for eight minutes + mercuric chloride (0.1%) for three minutes and sodium hypochlorite (4%) for nine minutes + mercuric chloride (0.1%) for five minutes in nodal segments and in shoot tips (96.67%) it was observed in combination of sodium hypochlorite (4%) for nine minutes + mercuric chloride (0.1%) for five minutes.

Table 4: Effect of different concentrations of mercuric chloride combined with best exposure timings of sodium hypochlorite (4%) on contamination (%) and health of explants of finger lime.

Exposure time of NaOCl	Concentration and exposure time of $HgCl_2$	Contamination (%)		Health of explants	
		Nodal segment	Shoot tip	Nodal segment	Shoot tip
0	0	100.00 (89.71)^{a}	100.00 (89.71)^{a}	+	+
8 min	(0.025%) 3 min	83.33 (65.91)^{b}	73.33 (58.91)^{b}	++	++
9 min	(0.025%) 5 min	76.67 (61.13)^{c}	70.00 (56.79)^{c}	++	++
10 min	(0.025%) 8 min	66.67 (54.74)^{d}	66.67 (54.74)^{d}	+++	+++
8 min	(0.05%) 3 min	63.33 (52.73)^{e}	56.67 (48.84)^{e}	+++	+++
9 min	(0.05%) 5 min	56.67 (48.84)^{f}	50.00 (45.00)^{f}	+++	+++
10 min	(0.05%) 8 min	53.33 (46.91)^{g}	46.67 (43.09)^{f}	+++	+++
8 min	(0.10%) 3 min	56.67 (48.84)^{f}	40.00 (39.23)^{g}	+++	+++
9 min	(0.10%) 5 min	53.33 (46.91)^{g}	36.67 (37.26)^{g}	+++	+++
10 min	(0.10%) 8 min	43.33 (41.16)^{h}	30.00 (33.21)^{h}	++	++
S.E m±		0.23	0.25	-	-
LSD at 0.01		1.93	2.02	-	-
CV		1.49	1.72	-	-

Table 5: Effect of different concentrations of mercuric chloride combined with best exposure timings of sodium hypochlorite (4%) on survival (%) of explants of finger lime.

Exposure time of NaOCl	Concentration and exposure time of $HgCl_2$	Survival (%)	
		Nodal segment	Shoot tip
0	0	0.00 (0.29)^{f}	20.00 (26.57)^{i}
8 min	(0.025%) 3 min	86.67 (68.63)^{d}	70.00 (56.79)^{h}
9 min	(0.025%) 5 min	93.33 (75.04)^{c}	73.33 (58.91)^{g}
10 min	(0.025%) 8 min	93.33 (75.04)^{c}	76.67 (61.13)^{f}
8 min	(0.05%) 3 min	96.67 (79.48)^{b}	83.33 (65.91)^{e}
9 min	(0.05%) 5 min	96.67 (79.48)^{b}	86.67 (68.63)^{d}
10 min	(0.05%) 8 min	100.00 (89.71)^{a}	90.00 (71.57)^{c}
8 min	(0.10%) 3 min	100.00 (89.71)^{a}	93.33 (75.04)^{b}
9 min	(0.10%) 5 min	100.00 (89.71)^{a}	96.67 (79.48)^{a}
10 min	(0.10%)	73.33	76.67

	8 min	(58.91)[e]	(61.13)[f]
S.E m±		0.16	0.23
LSD at 0.01		1.62	1.91
CV		0.99	1.32

Initiation of response in nodal segments was found to be 100 percent in all the treatments except control (Table 6). In shoot tips, initiation of response was comparatively lower to nodal segments. The days taken for shoot induction did not differ much with the treatments. In both nodal and shoot tip explants, the maximum shoot induction was found within 15 days (Table 6). The nodal segments of Rough lemon treated with sodium hypochlorite (5%) for 10 min followed by mercuric chloride (0.2%) for 10 min took about 9.05 days for shoot induction (Taye *et al*., 2018) [12].

Table 6: Effect of different concentrations of mercuric chloride combined with best exposure timings of sodium hypochlorite (4%) on initiation of response (%) and days taken for shoot induction in explants of finger lime.

Exposure time of NaOCl	Concentration and exposure time of $HgCl_2$	Initiation of response (%)		Days taken for shoot induction	
		Nodal segment	Shoot tip	Nodal segment	Shoot tip
0	0	66.67 (54.74)[c]	6.67 (14.97)[i]	0.00 (1.00)[c]	0.00 (1.00)[c]
8 min	(0.025%) 3 min	83.33 (65.91)[b]	13.33 (21.41)[h]	16.00 (4.11)[a]	14.67 (3.96)[ab]
9 min	(0.025%) 5 min	100.00 (89.71)[a]	16.67 (24.06)[g]	15.33 (4.04)[a]	15.00 (4.00)[a]
10 min	(0.025%) 8 min	100.00 (89.71)[a]	23.33 (28.86)[f]	15.00 (4.00)[a]	14.33 (3.92)[ab]
8 min	(0.05%) 3 min	100.00 (89.71)[a]	30.00 (33.21)[e]	14.33 (3.92)[ab]	14.33 (3.92)[ab]
9 min	(0.05%) 5 min	100.00 (89.71)[a]	36.67 (37.26)[d]	14.33 (3.92)[ab]	14.33 (3.92)[ab]
10 min	(0.05%) 8 min	100.00 (89.71)[a]	43.33 (41.16)[c]	14.33 (3.92)[ab]	14.67 (3.96)[ab]
8 min	(0.10%) 3 min	100.00 (89.71)[a]	50.00 (45.00)[b]	12.33 (3.65)[b]	13.33 (3.78)[b]
9 min	(0.10%) 5 min	100.00 (89.71)[a]	60.00 (50.77)[a]	12.33 (3.65)[b]	14.00 (3.87)[ab]
10 min	(0.10%) 8 min	100.00 (89.71)[a]	40.00 (39.23)[cd]	13.33 (3.78)[ab]	14.00 (3.87)[ab]
S.E m±		0.06	0.38	0.11	0.05
LSD at 0.01		0.96	2.49	0.43	0.20
CV		0.49	3.20	5.12	2.34

Effect of different exposure timings of ethanol (70%) combined with best treatments of mercuric chloride and sodium hypochlorite (4%) on explants of finger lime.

From the above findings, in this study, treatments sodium hypochlorite (4%) for ten minutes + mercuric chloride (0.05%) for eight minutes, sodium hypochlorite (4%) for eight minutes + mercuric chloride (0.1%) for three minutes and sodium hypochlorite (4%) for nine minutes + mercuric chloride (0.1%) for five minutes were considered as best in finger lime based on the highest survival percentage and lower contamination and these combined with the different exposure timings of ethanol (70%). Naturally, 70 percent ethanol with 30 percent water will osmotically enter the cell wall better than 100 percent ethanol alone since it is more polar. Nearly all types of living microbes on the surface of the explants can be eliminated by this concentration (Coté, 1998) [3]. In this study, the lowest contamination in nodal (6.67%) and shoot tip (3.33%) was noted in sodium hypochlorite (4%) for eight minutes + mercuric chloride (0.1%) for five minutes + ethanol (70%) for 45 seconds (Table 7). However, in this treatment survival (Table 8) and health of explants (Table 7) diminished mainly because of higher concentration and exposure timings caused lethal effects on the explants. It is well known that decreasing the sterilizing agent concentration while increasing exposure time will lower the rate of explant mortality during surface sterilization, and *vice versa* will reduce the sterilizing agents phytotoxic action. As shoot tips are tender compared to nodal segments, survival decreased with the increase in concentration of sterilants. The highest survival (100%) (Table 8) along with low contamination (10%) was recorded in treatment sodium hypochlorite (4%) for eight minutes + mercuric chloride (0.1%) for five minutes + ethanol (70%) 30 seconds in nodal segments whereas, in shoot tips sodium hypochlorite (4%) for nine minutes + mercuric chloride (0.1%) for three minutes + ethanol (70%) for 45 seconds was found effective in obtaining maximum survival (96.67%). Simlilarly, combination of three sterilants were used in strawberry nodal and leaf explants (Oo *et al*., 2018).

Table 7: Effect of different exposure timings of ethanol (70%) combined with best treatments of mercuric chloride and sodium hypochlorite (4%) on contamination (%) and health of explants of finger lime.

Exposure time of NaOCl	Concentration and exposure time of $HgCl_2$	Exposure time of ethanol	Contamination (%)		Health of explants	
			Nodal segment	Shoot tip	Nodal segment	Shoot tip
0	0	0	100.00 (89.71)[a]	100.00 (89.71)[a]	+	+
10 min	(0.05%) 8 min	15 sec	33.33 (35.25)[b]	33.33 (35.25)[b]	++	++
10 min	(0.05%) 8 min	30 sec	30.00 (33.21)[bc]	26.67 (31.08)[c]	+++	+++
10 min	(0.05%) 8 min	45 sec	23.33 (28.86)[d]	23.33 (28.86)[d]	+++	+++
9 min	(0.10%) 3 min	15 sec	33.33 (35.25)[b]	26.67 (31.08)[c]	+++	+++
9 min	(0.10%) 3 min	30 sec	30.00 (33.21)[bc]	(20.00 26.57)[de]	+++	+++
9 min	(0.10%) 3 min	45 sec	13.33 (21.35)[e]	16.67 (24.04)[ef]	+++	+++
8 min	(0.10%) 5 min	15 sec	26.67 (31.07)[cd]	13.33 (21.36)[fg]	+++	+++
8 min	(0.10%) 5 min	30 sec	6.67 (14.96)[f]	10.00 (18.44)[g]	+++	+++
8 min	(0.10%) 5 min	45 sec	6.67 (14.96)[f]	3.33 (10.51)[h]	++	++
S.E m±			0.61	0.75	-	-
LSD at 0.01			3.14	3.47	-	-
CV			4.01	4.72	-	-

Table 8: Effect of different exposure timings of ethanol (70%) combined with best treatments of mercuric chloride and sodium hypochlorite (4%) on survival (%) of explants of finger lime.

Exposure time of NaOCl	Concentration and exposure time of $HgCl_2$	Exposure time of ethanol	Survival (%)	
			Nodal segment	Shoot tip
0	0	0	0.00 (0.29)[e]	20.00 (26.57)[i]
10 min	(0.05%) 8 min	15 sec	73.33 (58.91)[d]	70.00 (56.79)[g]
10 min	(0.05%) 8 min	30 sec	76.67 (61.12)[c]	73.33 (58.91)[f]
10 min	(0.05%) 8 min	45 sec	100.00 (89.71)[a]	100.00 (89.71)[a]
9 min	(0.10%) 3 min	15 sec	73.33 (58.91)[d]	83.33 (65.91)[d]
9 min	(0.10%) 3 min	30 sec	100.00 (89.71)[a]	86.67 (68.63)[c]
9 min	(0.10%) 3 min	45 sec	100.00 (89.71)[a]	100.00 (89.71)[a]
8 min	(0.10%) 5 min	15 sec	90.00 (71.57)[b]	96.67 (79.48)[b]
8 min	(0.10%) 5 min	30 sec	100.00 (89.71)[a]	96.67 (79.48)[b]
8 min	(0.10%) 5 min	45 sec	73.33 (58.91)[d]	60.00 (50.77)[h]
S.E m±			0.02	0.15
LSD at 0.01			0.54	1.54
CV			0.35	0.98

In present study, the initiation of response and days taken for shoot induction (Table 9) was not much affected by the sterilization treatment when done in combination of three sterilants. The highest response was recorded in nodal segments (100%) whereas; shoot tips recorded 73.33 percent response. This may be due to tender nature of explants where buds are affected by sterilization treatment. The average days taken for shoot initiation ranged from 13.40 to 15. Debbarma *et al*. (2016) [4] reported that by treating the auxillary buds of grapevines with ethanol (70%) 30 sec followed by mercuric chloride (0.1%) sterilization for 10 min resulted in highest initiation of response (86%). Overall these findings suggested that combination of various surface sterilizing agents can be most effective to eliminate microorganisms in the explants yielding clean cultures.

Table 9: Effect of different exposure timings of ethanol (70%) combined with best treatments of mercuric chloride and sodium hypochlorite (4%) on initiation of response (%) and days taken for shoot induction in explants of finger lime.

Exposure time of NaOCl	Concentration and exposure time of $HgCl_2$	Exposure time of ethanol	Initiation of response (%)		Days taken for shoot induction	
			Nodal segment	Shoot tip	Nodal segment	Shoot tip
0	0	0	50.00 (45.00)^{c}	6.67 (14.97)^{f}	0.00 (1.00)^{b}	0.00 (1.00)^{c}
10 min	(0.05%) 8 min	15 sec	100.00 (89.71)^{a}	60.00 (50.77)^{e}	14.67 (3.96)^{a}	14.50 (3.94)^{ab}
10 min	(0.05%) 8 min	30 sec	100.00 (89.71)^{a}	63.33 (52.73)^{d}	15.00 (4.00)^{a}	15.00 (4.00)^{a}
10min	(0.05%) 8 min	45 sec	100.00 (89.71)^{a}	63.33 (52.73)^{d}	15.00 (4.00)^{a}	13.40 (3.79)^{b}
9 min	(0.10%) 3 min	15 sec	100.00 (89.71)^{a}	66.67 (54.74)^{c}	15.00 (4.00)^{a}	14.33 (3.92)^{ab}
9 min	(0.10%) 3 min	30 sec	100.00 (89.71)^{a}	73.33 (58.91)^{a}	14.33 (3.92)^{a}	14.33 (3.92)^{ab}
9 min	(0.10%) 3 min	45 sec	100.00 (89.71)^{a}	73.33 (58.91)^{a}	14.33 (3.92)^{a}	14.33 (3.92)^{ab}
8 min	(0.10%) 5 min	15 sec	100.00 (89.71)^{a}	70.00 (56.79)^{b}	14.33 (3.92)^{a}	14.00 (3.87)^{ab}
8 min	(0.10%) 5 min	30 sec	100.00 (89.71)^{a}	70.00 (56.79)^{b}	14.00 (3.87)^{a}	13.33 (3.78)^{b}
8 min	(0.10%) 5 min	45 sec	96.67 (79.48)^{b}	70.00 (56.79)^{b}	14.67 (3.96)^{a}	14.17 (3.89)^{ab}
S.E m±			0.05	0.11	0.06	0.04
LSD at 0.01			0.85	1.35	0.23	0.15
CV			0.44	1.13	2.72	1.82

Conclusion

In conclusion, our investigation into surface sterilization protocols for initiating *in vitro* cultures of finger lime (*Citrus australasica F. Muell*) has yielded critical insights essential for the sustainable production of this unique citrus. The optimization of sodium hypochlorite exposure, both independently and in combination with mercuric chloride, has proven instrumental in minimizing contamination while ensuring the robust initiation of cultures.

Our findings underscore the importance of nodal segments as the most responsive explants, exhibiting a 100% initiation response across various sterilization treatments. This insight guides the selection of optimal explants types for initiating finger lime cultures efficiently. Additionally, the introduction of a brief ethanol treatment further enhances the sterilization process, contributing to reduced contamination levels, particularly in nodal and shoot tip explants.

The current study also reported that instead of using single surface sterilizing agent it is most advocated to use in combination. The combination of sodium hypochlorite (4%) for eight minutes + mercuric chloride (0.1%) for five minutes + ethanol (70%) for 30 seconds is the common best sterilization procedure for both nodal and shoot tip explants based on the observations recorded and it can be used in further studies.

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