



Original Research Article

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In vitro Sterilization Protocol for Establishment of Litchi (*Litchi chinensis* Sonn) cv. Purbi

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Litchi is considered a crop difficult to propagate through micropropagation and obtaining contamination free cultures is first and foremost requirement of tissue culture. So the present investigation was carried out to standardize the sterilization procedure of nodal segment and leaf explants of *Litchi chinensis* Sonn. cv. Purbi. Two surface sterilizing agents viz. HgCl₂ and NaOCl were used at varying concentrations and durations. In this experiment HgCl₂ was found to be better sterilizing agent than NaOCl for both the explants. HgCl₂ (0.2 %) for 3 min treatment was found to be the most effective for nodal segment explants that resulted in maximum per cent survival ($56.5\pm1.19\%$), low percent mortality ($13.8\pm0.18\%$) and low per cent contamination ($33.1\pm0.51\%$). While for leaf explants HgCl₂ (0.1 %) for 1.0 min yielded best results with highest per cent survival ($53.9\pm1.08\%$), low per cent mortality ($14.0\pm0.21\%$) and low per cent contamination ($35.5\pm0.56\%$).

Introduction

Litchi (*Litchi chinensis* Sonn.) is one of the most popular subtropical fruits highly prized for its luscious white arils and has been rightly called as “Queen of fruits”. It requires highly specific climate which directly affects its quality characters like shape, size, texture, and nutritive value. It belongs to the

Sapindaceae or soapberry family and sub family Nepheleae which comprises not less than 150 genera and 2000 species. It is grown as a major commercial crop in China, Taiwan, Vietnam, Thailand, India, Madagascar, South Africa and Reunion Islands and to a limited extent in Australia, New Zealand, Indonesia, Mauritius, Israel, Spain, the U.S and Mexico. China has litchi map of the world both, in

terms of production and productivity. Conventionally litchi is propagated by vegetative means mainly through air layering or marcottage. Although various means to increase the efficiency of this method have been tried such as, use of younger branches, small earth balls and 1,4-indole-3-butyric acid (IBA), the process is still slow and inefficient. Hence, for large scale production of elite litchi clones, micropropagation can be used as a potential alternative to the vegetative means of reproduction. However, till date litchi has been proved to be a difficult material to be propagated using *in vitro* culture.

Plant tissue culture is a system of growing plant cells, tissue or organs, that have been separated from the mother plant (called explants) in artificial medium under aseptic condition (Omamor *et al.*, 2007). Even though aseptic conditions are maintained, plant cultures may not stay aseptic in *in vitro*. The *in vitro* culture of any cell, organ and tissue can be contaminated basically from 4 types of sources. These sources are the plant (internal as well as external), the nutrient medium (insufficiently sterilized), the air, and the research worker (improper sterile techniques) (Pierik, 1994; Urbi and Zainuddin, 2015). Out of these, contamination resulting from microorganisms already present in the explant is a major challenge to establishment, initiation and maintenance of aseptic *in vitro* cultures.

The plants when grown under field conditions often get contaminated with a lot of soil and air borne pathogens and it therefore necessitates a thorough and effective sterilization procedure of the explants before culturing. The aim of this study was to investigate and identify the most effective sterilization technique for nodal segment and leaf explants of *Litchi chinensis* obtained from the field.

Materials and Methods

Two sterilizing agents or disinfectants namely NaOCl and HgCl₂, with different concentrations and various exposure time were used to surface sterilize the explants collected from the field. Litchi cultivar Purbi grown at Horticulture Garden of Bihar Agricultural College, Sabour was selected for the present investigation as source of explants. For the *in vitro* establishment, the required plant material used was leaf and nodal segment. Young shoot branches were cut from the healthy and disease free plants of selected genotype of litchi. They were brought to the laboratory and nodal segments containing axillary bud were cut out using scalpel and forceps, of about 1-2 cm length. Whereas, the young leaves excised in the form of 1 cm × 1cm were also collected as explants. Firstly, the prepared explants in suitable sizes were washed in running tap water 4-5 times. The washed explants were then washed in a solution containing 2- 3 drops detergent (tween -20) and 1-2 ml dettol for about 10 minutes. Thereafter explants were washed 2-3 times with sterilized water. The cleaned out nodal segment and leaf explant were then dipped in 0.2% bavistin solution for 50 minutes and 30 minutes respectively to control the fungal contamination. The nodal segments were then pretreated in a solution of 0.4 % ascorbic acid for 40-50 minutes and leaves in 0.1% ascorbic acid solution for an hour in a beaker. This pretreatment with ascorbic acid was done to control phenolic exudation from the wounded parts. It also resulted in reduced microbial contamination. The pretreated explants were washed at least 3 times with sterile distilled water under laminar air flow. For surface sterilization these pretreated nodal explants were then treated with 0.1% and 0.2% HgCl₂ for 1,2,3,4 and 5 minutes and 1.0% NaOCl for 2, 4, 6, 8 and 10 min while treatment of HgCl₂ for 0.25, 0.5, 0.75, 1.0, 1.25, and 1.5 minutes

and NaOCl for 1, 2, 3, 4, 5 and 6 minutes was given to leaf explants.

Results and Discussion

The efficiency of the surface sterilants was evaluated based on the number of live aseptic cultures.

Effect of surface sterilants on nodal segment explants

100% contamination was observed when no sterilant treatment was given. The contamination percent reduced with the increase of concentration and time duration of HgCl₂. Minimum contamination of 21.4 (27.5±0.40) % was recorded when 0.2% HgCl₂ treatment was given for 5 mins. Although this treatment decreased contamination, but at the same time also

caused maximum mortality 41.2 (39.9±0.65) % as compared to all other treatments. However, 0.2% HgCl₂ for 3min resulted in maximum survival 69.5 (56.5±1.19) % of nodal segment explants with mortality of only 5.7 (13.8±0.18) % explants and contamination of 29.8 (33.1±0.51) % explants. Sodium hypochlorite on the other hand although reduced the mortality rate but the efficiency to control contamination was much lower than various treatments of HgCl₂. Although per cent contamination was seen negatively correlated with the concentration and time of exposure of the sterilants, the survival percent significantly reduced. Overall, HgCl₂ (0.2) % for 3 min treatment was found to be the most effective that resulted in maximum per cent survival (56.5±1.19)%, low percent mortality (13.8±0.18)% and low per cent contamination (33.1±0.51)%.

Table 4.1 Effect of different treatment and duration of surface sterilants on nodal explants of litchi cv. Purbi

Treatment	Treatment and duration	Per cent contamination	Per cent mortality	Per cent survival
T0	Control	100.0 (90.0±0.00)	0.0 (0.0±0.00)	0.0 (0.0±0.00)
T1	HgCl ₂ (0.1 %) for 1 min	98.2 (83.0±2.25)	1.5 (6.9±0.08)	4.6 (12.4±0.16)
T2	HgCl ₂ (0.1 %) for 2 min	94.6 (76.5±0.37)	2.2 (8.5±0.11)	8.2 (16.6±0.24)
T3	HgCl ₂ (0.1 %) for 3 min	90.9 (72.8±2.59)	2.8 (9.7±0.15)	11.2 (19.6±0.29)
T4	HgCl ₂ (0.1 %) for 4 min	88.8 (70.7±2.28)	3.4 (10.6±0.14)	12.8 (20.9±0.30)
T5	HgCl ₂ (0.1 %) for 5 min	82.4 (65.3±1.73)	5.4 (13.4±0.18)	17.2 (24.5±0.35)
T6	HgCl ₂ (0.2%) for 1 min	58.4 (49.8±0.94)	4.4 (12.1±0.16)	42.2 (40.5±0.67)
T7	HgCl ₂ (0.2%) for 2 min	38.1 (38.1±0.61)	5.6 (13.6±0.18)	61.3 (51.5±0.98)
T8	HgCl ₂ (0.2 %) for 3 min	29.8 (33.1±0.51)	5.7 (13.8±0.18)	69.5 (56.5±1.19)
T9	HgCl ₂ (0.2 %) for 4 min	27.8 (31.8±0.50)	19.0 (25.8±0.38)	58.2 (49.7±0.92)
T10	HgCl ₂ (0.2%) for 5 min	21.4 (27.5±0.40)	41.2 (39.9±0.65)	42.4 (40.6±0.67)
T11	NaOCl (1%) for 2 min	89.7 (71.5±2.36)	7.2 (15.6±0.22)	8.1 (16.5±0.24)
T12	NaOCl (1%) for 4 min	84.4 (66.8±1.84)	8.6 (17.0±0.24)	12.0 (20.2±0.28)
T13	NaOCl (1%) for 6 min	76.0 (60.7±1.40)	11.7 (19.9±0.28)	17.3 (24.6±0.37)
T14	NaOCl (1%) for 8 min	69.7 (56.6±1.19)	11.8 (20.0±0.28)	23.5 (29.0±0.43)
T15	NaOCl (1%) for 10 min	63.4 (52.8±1.03)	12.8 (20.9±0.30)	28.8 (32.4±0.49)
C.D		4.30	0.76	1.65
S.E(m)		1.49	0.26	0.57

Table.2 Effect of different treatment duration of sterilizing agents on leaf explants

Treatment	Treatment and duration	Per cent contamination	Per cent mortality	Per cent survival
T₀	Control	100.0 (89.4±0.62)	0.0 (0.0±0.00)	0.0 (0.0±0.00)
T₁	HgCl ₂ (0.1 %) for 0.25 min	84.6 (67.0±1.87)	3.8 (11.2±0.17)	16.6 (24.0±0.36)
T₂	HgCl ₂ (0.1 %) for 0.5 min	52.9 (46.6±0.83)	4.1 (11.7±0.17)	48.0 (43.8±0.76)
T₃	HgCl ₂ (0.1 %) for 0.75min	47.0 (43.3±0.75)	5.0 (12.9±0.19)	52.9 (46.6±0.83)
T₄	HgCl ₂ (0.1 %) for 1.0 min	33.8 (35.5±0.56)	5.9 (14.0±0.21)	65.3 (53.9±1.08)
T₅	HgCl ₂ (0.1 %) for 1.25 min	30.2 (33.3±0.52)	16.1 (23.6±0.34)	58.7 (50.0±0.94)
T₆	HgCl ₂ (0.1%) for 1.5 min	25.7 (30.5±0.47)	29.2 (32.7±0.51)	50.1 (45.0±0.79)
T₇	NaOCl (1%) for 1 min	95.8 (78.9±3.83)	2.5 (9.1±0.11)	6.7 (15.0±0.20)
T₈	NaOCl (1%) for 2 min	84.7 (67.1±1.88)	2.8 (9.7±0.15)	17.4 (24.7±0.37)
T₉	NaOCl (1%) for 3 min	71.9 (58.0±1.27)	4.4 (12.1±0.16)	28.7 (32.3±0.49)
T₁₀	NaOCl (1%) for 4 min	63.1 (52.6±1.03)	5.7 (13.8±0.18)	36.2 (37.0±0.60)
T₁₁	NaOCl (1%) for 5 min	55.3 (48.0±0.88)	6.0 (14.2±0.21)	43.7 (41.3±0.70)
T₁₂	NaOCl (1%) for 6 min	48.1 (43.9±0.76)	14.4 (22.3±0.33)	42.5 (40.7±0.69)
C.D		4.30	0.70	1.95
S.E(m)		1.47	0.24	0.67

Effect of surface sterilants on leaf explants

As the concentration and duration of treatments were increased contamination decreased but it also led to the aggravation in mortality rate (table 2). 100% explants were contaminated in control conditions. The contamination percent of explants decreased with increasing time of exposure for both the sterilants. But with the increasing time duration the mortality percent also increased while the survival percent decreased. Although treatment T₆ showed least contamination of 25.7 (30.5±0.47)% but it also showed a mortality of 29.2 (32.7±0.51)% which was highest compared to other treatments. T₄ was found to be the most effective treatment resulting in highest per cent survival (53.9±1.08) %, low per cent mortality (14.0±0.21) % and low per cent contamination (35.5±0.56) %.

The use of field grown plants as direct sources of explants for the production of ‘clean’ *in vitro* plantlets, presents a major challenge (Webster *et al.*, 2003). This is

because the surface of field grown plants carries a wide range of microorganisms (Daud *et al.*, 2012). Nearly all fungal and yeast and some bacterial species are severe hazards *in vitro* because they grow well on plant tissue culture media thus increasing the competition for nutrients and kill plants by reducing the pH and the production of toxic metabolites (Leifert *et al.*, 1991). So, surface sterilization is a must before transfer of explants to the culture media. In this experiment we concluded that exposure to lower concentration of sterilants, increased the contamination of explants, whereas exposure to higher concentrations for longer duration though reduced the contamination but also increased the mortality considerably for all the explants. This indicates the deleterious effect of the sterilants at higher concentrations. In this experiment HgCl₂ was found to be better sterilizing agent than NaOCl for both the explants. The effectiveness of HgCl₂ for surface sterilization of explants from woody plants has been reported by several workers such as Chandra *et al.*, (2004) in mango, Zamir *et al.*, (2004) in guava, Daud *et al.*, (2012) in *Aquilaria*

malaccensis and Parveen *et al.*, (2019) in pineapple.

In conclusion, the results of the present study showed that among the two surface sterilizing agents tested, HgCl₂ was better than NaOCl for both the explants i.e. nodal segments and leaves. Also it was found that although increasing the concentration and duration of exposure to sterilizing agents beyond certain limits reduced contamination but at the same time it also increased the mortality percent of the explants

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