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Effect of Surface Sterilants on *in vitro* Establishment of Pineapple (*Ananas comosus* (L.) Merill.) cv. Kew

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Authors' contributions

This work was carried out in collaboration between all authors. Author SP carry out the study, developed protocol for establishment of pineapple and wrote the first draft of the manuscript. Author HM designed the study, performed the statistical analysis and edited the first draft and authors TR and AKP provided the valuable guidance during course of study. Author MK managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

The rate of pineapple propagation through conventional technique is quite low and time taken and that by seed is apparently hard to germinate. Non-availability of quality planting material is one of the major constraints for expansion of its cultivation area in Bihar. Keeping this in view an experiment was conducted for *in vitro* establishment for large scale disease free planting materials production. The most commonly encountered problem during *in vitro* pineapple germplasm establishment is the rate of contamination, which is very high in case of pineapple. Suckers of pineapple cultivar Kew were used as explants for the study. In this experiment the efficiency of three sterilizing agents (Clorox, $HgCl_2$ and NaOCl) at different concentrations and duration was evaluated

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in terms of number of aseptic cultures. Results revealed that when no sterilant was used all the cultures were contaminated. The contamination of explants significantly decreased with increase in concentration of different sterilants and their time of exposure. The highest survival of explants (58.31±1.71%) were observed when explants were treated with Clorox 40% for 20 minutes which also resulted in 17.89±0.25% and 25.03±2.63% mortality and per cent contamination respectively. As the duration of Clorox 40% was increased, percent contamination decreased but simultaneously increased the mortality rate at 25 mins of duration. The percent survival also increased when explants were treated with different concentrations of $HgCl_2$ and NaOCl at different time durations. However, NaOCl treatments were less effective as compared to other two sterilants.

Keywords: Surface sterilants; in vitro establishment; pineapple; propagation.

1. INTRODUCTION

Pineapple (*Ananas comosus* L. Merr.) is a perennial monocotyledonous herbaceous fruit crop of Bromeliaceae family, grows to the height of 1.0 to 1.5 m (3.3 to 4.9 ft) although sometimes can be more tall. It is one of the commercially important tropical fruit crops and major exported commodity from India [1]. Taxonomy of the genus *Ananas* is poorly characterized [2,3,4]. The basic chromosome number of Pineapple is (2X=50). Although it is diploid, some triploid and tetraploid types have also been reported [5]. Pineapple on an average produces two propagules/ year. Under ideal conditions with optimum water supply and no disease-pest infestation, pineapple takes about 24 to 36 months to yield fruits and also multiplication of pineapple through slips and suckers are highly laborious, have very low multiplication rate and a slow regeneration cycle of new suckers [6]. The most important pineapple variety cultivated in Bihar is Kew. Large numbers of propagules required for commercial cultivation of the Kew variety in order to meet the farmers demand in the state cannot be obtained via traditional propagation through slips, suckers and crowns. Hence, for mass production of planting material round the year, micro propagation can be used as potential alternative. It not only provides a crucial advantage over the conventional propagation method but also improved performances are being observed in the same variety for different parameters. Micropropagated plants yield higher and have uniform maturity. It is also possible to produce 12.5 lakh plantlets of pineapple only with 30 explants [7]. However, for successful *in vitro* propagation removal of contamination is necessary. Without successful sterilization it is difficult to investigate the effect of chemical and physical factors of medium on the establishment of primary explants and most of the explant would be lost and we cannot even start the planned project. The growth and development of successfully sterilized explant also depends on other factors such as medium types [8,9].and states [10,11], hormone types [12,13] concentrations [14] and combinations [15,16], explants dryness and cultivars [12]. Keeping in view all these factors, the present work was planned to study the effect of surface steriliants on *in vitro* establishment of pineapple *(Ananas comosus* l. Merill.) cv. Kew

2. MATERIALS AND METHODS

This investigation was carried out in plant tissue culture laboratory, Bihar Agricultural College, Sabour, Bhagalpur during 2017-18. Suckers of Pineapple cultivar “Kew” grown in the farmer’s field of Kishanganj district of Bihar were collected and used as explants for the investigation of *in vitro* establishment. The leaves were removed and the suckers were washed under running tap water to remove dust and dry matters and then treated with teepol. Explants were then treated with 0.2 per cent bavistin for 30 minutes and were washed 3-4 times with distilled water. The washed explants were surface sterilized in the laminar air flow with three different steriliants namely $HgCl_2$ (0.1%), Clorox (40%) and NaOCl (2%) each with five different time duration (5, 10,15,20 & 25 mins) and finally comprise total 15 treatments (Table 1). The explants were given 5-6 washing with sterilized distilled water to remove the traces of sterilizing agent. The surface sterilized and aseptically excised explants were finally placed on the prepared media by working in a laminar air flow cabinet. During inoculation the explants were properly positioned on the media and were gently pressed with forceps to secure their firm contact with the media. The treated explants were cut aseptically and cultured on shoot proliferation media containing various combinations of growth regulators. The recorded data was analysed with CRD (Completely Randomized Design) with three replications and 3 units (bottles) were maintained

for each replication. The significance of variance among the treatments was observed by applying 'F' test and critical difference at 5 per cent level of significance to compare the treatments for all the characters. The percent data in sterilization were transformed by angular transformation before the statistical analysis.

3. RESULTS AND DISCUSSION

For successful culture, establishment of an aseptic technique is a pre requisite. The plants when grown under field conditions often get contaminated with lot of soil and air borne pathogens and it therefore necessitates a thorough and effective sterilization procedure of the explants before culturing. In this study explants were collected from experimental field, where contamination is usually considered to be a serious problem and surface sterilization becomes a crucial task. Since large percentage of contamination was observed of fungal origin, treatment with bavistin @ 0.2 percent for half an hour proved effective for reducing fungal infection of the cultures. The data regarding the effect of different treatment duration of surface sterilants on pineapple cultivar Kew explant is presented in Table 1 and Fig. 1. The efficiency of the surface sterilants was evaluated based on the number of live aseptic cultures. In this establishment experiment, three different types of sterilant were used at different concentration and time duration and the percent contamination, percent mortality and percent survival were recorded. All the cultures were contaminated when no sterilant treatment was given to explants. Although percent contamination decreased significantly with the concentration and time of exposure of the sterilants, the survival percent reduced after certain concentrations. The highest survival percentage of explants (58.31±1.71) were observed in treatment T9 when explants were treated with Clorox 40% for 20 mins while (17.89±0.25) and (25.03±2.63) were the percent mortality and perecent contamination respectively observed in the same treatment. It was evident from data that as the duration of Clorox 40% was increased, percent contamination decreased but it also increases the mortality rate at 25 mins. Data also showed that as compared to control, the percent survival rate of cultures increased when explants were treated with different concentrations of $HgCl_2$ and NaOCl at different time durations. Among all the $HgCl_2$ treatments, the highest survival (44.74±1.25%) were recorded at treatment T2 when explants were treated with $HgCl_2$ 0.1% for 10 mins while (14.65±0.21) and (41.53±2.07) percent mortality and percent contamination were noticed respectively in the same treatment. The minimum percent contamination (14.94±3.96) was observed in treatment T5 where longer exposure of $HgCl_2$ 0.1% at 25 minute were used but it also reduced the percent survival rate (21.98±1.80) and increased the percent mortality rate (62.29±1.50). NaOCl and $HgCl_2$ is highly antimicrobial have strong action against both fungi and bacteria. Although it is toxic at higher concentration but it is broad spectral antifungal. Use of NaOCl and Clorox at low concentration and frequent washing 4-5 times can reduce the toxicity problem. $HgCl_2$ is phytotoxic and has antibacterial properties, and can be more injurious to plant tissue. Many rinses are required to remove all traces of the mineral from the plant material. Exposure for longer duration of $HgCl_2$ damage and kills the plant tissue. So exposure at lower concentration for less time duration is recommended by many workers [17].

However NaOCl treatments were less effective as compared to other mentioned sterilants. Among NaOCl treated explants, maximum survival percentage (40.87±1.20) was recorded at when explants were treated for 20 mins. It has been observed that as the time duration of NaOCl (2%) was increased, mortality rate also increased while percent contamination decreased. But the percent survival (38.23±1.18) decreased when explants were treated for higher time duration of 25 minutes which again shows the toxic effect of NaOCl after a certain concentration.

Among the three different sterilizing agents used, Clorox was found to be better than mercuric chloride and sodium hypochlorite for maximum establishment of cultures. Successful sterilization and growth of explants is an essential key step which depends on different type of Sterilants and its concentration [18]. [19] has also suggested both clorox and $HgCl_2$ for surface sterilization. He used 10, 20, 30 and 40% Clorox for 20 mins while [20] sterilized the pineapple crown with clorox 20% for 25 mins.

Single and double steps procedure using Clorox and $HgCl_2$ were also reported by other workers for successful sterilization of terminal and lateral buds from suckers [21], shoots [22] and slips of pineapple [23]. Single step sterilization leads to 22% clean explants when Clorox (10%) for 15 minutes or HgCl2 (0.1%) for 5 minutes were

used. The clean explants percentage were increased by about three times (22 to 56%) by using Clorox concentration of 25% for 25 minutes. However double step sterilization using Clorox 25% for 25 minutes followed by either Clorox 10% for 15 minutes or $HgCl_2$ at 0.1% for 5 minutes have reduced the percentage of clean explants from 56 to 33%. This rate were again reduced by about three times (i.e 56% to 22%) when triple sterilization made of Clorox (25%) for 25 minutes, HgCl2 (0.1%) for 5 minute followed by Clorox (10%) for 15 minutes were used [24].

Table 1. Effect of different concentrations and durations of different surface sterilants on contamination, mortality and survival rate in pineapple cv. Kew

Treatment	Concentrations (%) and duration (Mins)	Contamination (%)	Mortality (%)	Survival (%)
T0	Control	100.00(90.00±0.00)	0.00(0.00±0.00)	0.00(0.00±0.00)
T1	$HgCl_2$ (0.1%) for 5 mins	70.38(57.10±2.47)	23.52(29.00±0.44)	6.10(13.98±2.21)
T2	$HgCl_2$ (0.1%) for 10 mins	44.02(41.53±2.07)	6.40(14.65±0.21)	49.57(44.74±1.25)
T3	$HgCl_2$ (0.1%) for 15 mins	39.30(38.77±2.09)	15.44(23.12±0.34)	45.27(42.26±1.21)
T4	$HgCl_2$ (0.1%) for 20 mins	13.05(20.78±3.10)	51.66(45.93±0.81)	35.29(36.42±1.17)
T5	$HgCl_2$ (0.1%) for 25 mins	7.50(14.94±3.96)	78.33(62.29±1.50)	14.17(21.98±1.80)
T6	Clorox (40%) for 5 mins	86.55(68.99±3.71)	2.00(8.12±0.11)	11.46(19.67±1.59)
T7	Clorox (40%) for 10 mins	70.48(57.17±2.47)	2.42(8.94±0.12)	27.10(31.34±1.19)
T8	Clorox (40%) for 15 mins	51.69(45.95±2.10)	3.57(10.89±0.15)	44.74(41.96±1.21)
T9	**Clorox (40%) for 20 mins**	**18.19(25.03±2.63)**	**9.45(17.89±0.25)**	**72.36(58.31±1.71)**
T10	Clorox (40%) for 25 mins	13.89(21.53±3.00)	19.11(25.91±0.38)	67.00(54.95±1.55)
T11	NaOCl (2%) for 5 mins	93.69(77.22±5.12)	1.99(8.11±0.11)	4.32(11.73±1.82)
T12	NaOCl (2%) for 10 mins	75.84(60.69±2.70)	2.31(8.74±0.12)	21.85(27.82±1.25)
T13	NaOCl (2%) for 15 mins	65.34(53.97±2.32)	3.25(10.39±0.14)	31.41(34.05±1.17)
T14	NaOCl (2%) for 20 mins	53.79(47.16±2.12)	3.36(10.56±0.15)	42.85(40.87±1.20)
T15	NaOCl (2%) for 25 mins	45.39(42.32±2.08)	16.28(23.78±0.35)	38.34(38.23±1.18)
C.D		8.18	1.40	4.11
CV		10.27	4.33	7.60

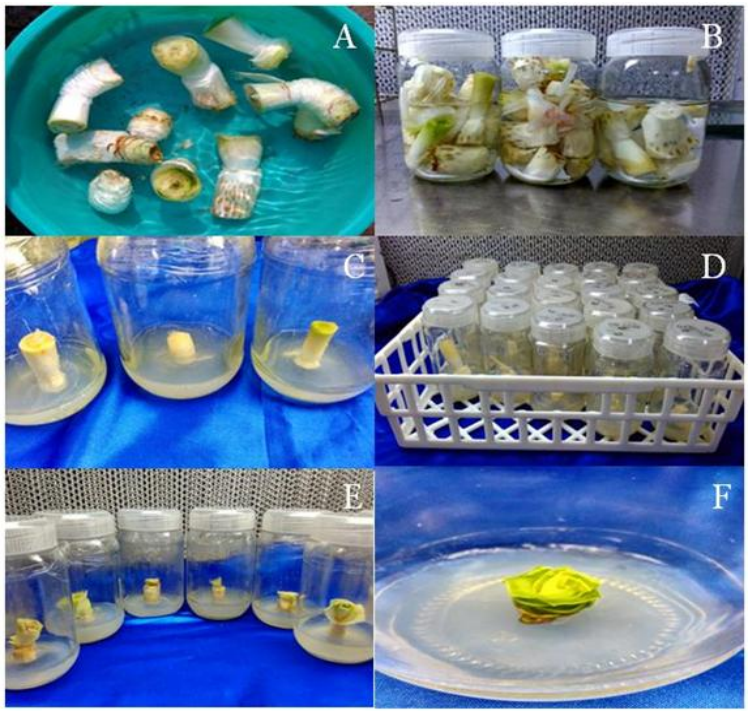

Fig. 1. Different stages of sterilants treatment during establishment of pineapple germplasm under *in vitro* condition

Sterilization and establishment are bottleneck phase of any tissue culture system and more efforts are needed to develop efficient and reproducible sterilization procedure. Many researchers have largely used NaOCl at different concentration and different duration for sterilizing explant [13]. According to [25] have surface sterilized the crown by NaOCl @ 10% in 20 min and then 5% in 10 min. Nelson et al. [26] used lower concentration of NaOCl @ 2% for 10, 15, 20, and 25 minute and found that at 25 minute all the culture were contamination free but the regenerations were very low compared to duration of 10, 15, 20 and he also observed that treatment at the same concentration for 10 minute give 55% non contaminated culture. However, prolonged sterilization leads to increase in mortality rate. The negative effect of longer exposure was also reported by many scientists [27,28,29]. Obtaining of uncontaminated explants does not guarantee its establishment. Using improper hormone concentration could also diminish or even block establishment of pineapple explants [12,14].

4. CONCLUSION

Clorox 40% for 20 mins proved very effective for sterilization and establishment of pineapple suckers. This also appeared to be a very reliable and cost effective protocol for initiating pineapples cultures.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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