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Sterilization and establishment of pineapple shoot tip

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Abstract.

Out of eight sterilization treatments made of combinations of Mercuric chloride ($HgCl_2$), Clorox and Ethanol at different concentrations and exposure times, Clorox (25%) for 25 minutes was the best treatment resulting in successful sterilization of 56 % of crown tips of Smooth cayenne pineapple. The sterilized crown tip grew and produced multiple shoots on agar solidified full strength MS medium enriched with 6-benzyleaminopurine (BAP) at four different concentrations (2.25, 3.25, 4.25 and 5.25 mg l^{-1}), but at different shoot formation rate. After 60 days of incubation, the highest shoot formation (7 shoots / tip) obtained in medium enriched with BAP at 2.25 mg l^{-1} followed by BAP at 3.25 mg l^{-1} (4 shoots). BAP at 4.25 and at 5.25 mg l^{-1} resulted in formation of 3 and 2 shoots per tip. The low percentage of successfull sterilization of crown tips (56 %) indicated that elimination of microbial ccontamination is still a serios problem and main drawback that prevent full investigation and optimization of establishment of crown tips of pineapple. Nevertheless, the few successfully sterilized crown tip proliferated into shoots good enough to investigate the next multiplication stage.

Key words: BAP; *Ananas comosus*; Sterilization; Establishment; Contamination; Clorox.

Introductrion.

Although, successful *in vitro* culture of pineapple have been reported in several journals, the focus was mainly on multiplication of the secondary explants while little attention was paid to establishment of the primary explants. The sterilization procedure and establiushment of primary explants was in most time vaguely and briefly reported and the claimed successful sterilization percentage of primary explants was irreproducible. The most commonly used sterilization agents are Clorox (house bleach with 5.25 % active chloride) and mercury chloride ($HgCl_2$) at 0.1%. Single and double steps procedure using Clorox and $HgCl_2$ were reported for sterilization of terminal and lateral buds from suckers (Sripaoraya *et al*., 2003; Bhatia and Ashwath, 2002; Kofi and Adachi, 1993; Liu *et al*., 1989; Fernando, 1986), shoots (Teng, 1997) and slips of pineapple (Almeida *et al*., 2002). Wakasa, (1989) reported that successful sterilization depended on explants types and Clorax concentration. Most of the reported sterilization procedure was made of one agent and concentration (Singh and Manual, 2000; Broomes and MacEvan, 1994; Zepeda and Sagawa, 1981) and no comparison were made between agents, concentrations and exposure times and between combination of agents and concentrations. Successfull sterilization and growth of explants is an essential key step. If all explants were contaminated or remained dormant, the planed project or experiments could not even be started. Without successfull sterilization, the effect of chemical and physical factors of medium on the establishment of primary explants would be difficult to investigate and optimize. Simply because if an experiment in which all of the factors that expected to effect establishment of primary explants was layed out most of the explants would be lost due to contamination.

Establishment means that the primary explants is being both microbial free and capable of sprouting, growth and development into strong solitary shoot, cluster of few shoots or callus. The growth and development of those primary explants that were successfully sterilized depended on so many other factors such as medium types (Liu *et al*., 1989; Bordoloi and Sarma, 1993) and states (Soneji, *et al.*, 2002; Broomes and MacEvan, 1994), hormone types (Devi *et al.*, 1997; Fitchet, 1990), concentrations (Bhatia and Ashwath, 2002) and combinations (Rahman *et al.*, 2001; Hirimburegama and Wijesinghe, 1992), explants dryness and cultivars (Fitchet, 1990), explants intactness and hot water treatment (Broomes and MacEvan, 1994) that need to be optimized. The consequence of obtaining low percentage of successfully established primary explants, would be a too long lag time before enough shoots become available for starting commercial propagules production (Dewald *et al.*, 1988). Large number of successfully established primary, on the other hand, would reduce the number of multiplication cycles required to produce the planed amount of propagules with minimum somaclonal variation (Smith *et al.*, 2002) and facilitate investigation and optimization of primary explants establishment. The objective of this study was firstly to test the effect of eight different sterilization procdures in which Clorax at different concentrations and exposure times and mercury chloride were use in single, double and triple steps of sterilization. Secondly to test the effect of four different concentrations of BAP (2.25, 3.25, 4.25 and 5.25 mg l^{-1}) on the establishment and shoot formation of crown tip of Smooth cayenne pineapple.

Materials and Methods.

Sterilization.

Crowns of Smooth cayenne were collected from public market, left to dry for one day and then defoliated and washed under running tap water for a half hour. The crown tip was trimmed to 1.0 cm^3 and soaked in solutions of different sterilants at different concentrations and exposure times as follow:

1- Clorox (10 %) for 15 minutes

2- Clorox (25 %) for 25 minutes

3- $HgCl_2$ (0.1 %) for 5 minutes

4- $HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) for 15 minutes

5- Ethanol (50 %) for 5 minutes; Clorox (10 %) for 15 minutes

6- Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute

7- Clorox (25 %) for 25 minutes; Clorox (10 %) for 15 minutes

8- Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) 15 minutes

Under laminar flow cabinet, the sterilants were decanted and the explants rinsed in sterilized distilled water for three times, placed in autoclaved petri dish and trimmed to 0.5 cm^3. Then the explants were cultured individually in glass jars (15 x 5 cm.) containing 20 ml of agar solidified (7.0 g/l) full strength MS medium (Murashige and Skoog, 1962) enriched with sucrose at 30 g/l. The cultures were kept in a culture room under 16 hours of light and 8 hours of darkness and constant temperature 24 ^{0} C for 45 to 60 days. The number of uncontaminated (growing and nongrowing) and contaminated but growing explants was recorded every week for 45 days. The sterilization experiment repeated two times using at each time 4 to 9 explants per each treatment.

Establishment.

Full strength MS medium was prepared from stock solutions enriched with sucrose at 30 g/l and divided into 4 glass jars (180 ml each). The medium in each jar was enriched with 6-benzyleaminopurine (BAP) at 2.25, 3.25, 4.25 and 5.25 mg/l, adjusted to pH 5.7 and dispensed into 9 jars (20 ml each). 0.14 grams of agar was added to each jar and the media were autoclaved at 121^0 C and 1.5 kg/ cm^2 for 25 minutes. Smooth cayenne crown tips were sterilized in Clorox at 25 % for 25 minutes, rinsed in sterilized distilled water, trimmed to 0.5 cm^3 and cultured individually one shoot tip per jar. The cultures were kept in a culture room under a photoperiod of 16 hours of light and constant temperature 24 ^{0} C. The cultures were observed weekly and the percentage of clean and growing, clean nongrowing and contaminated but growing explants were recorded. The number of shoots per culture was counted after 60 days of incubation. The experiment was repeated several times to get at least a total of 9 cultures of clean (growing, nongrowing or contaminated but growing) explants per each hormone treatment. Data were arranged in table of three replicates and subjected to

analysis of variance and Duncan Multiple Range Test at p 0.05 using SPSS statistical package No.11.

Results.

Soaking the crown tips on Clorax (25 %) for 25 minutes was the best of the tested sterilization methods. It resulted in 56 % clean and growing cultures of Smooth cayenne crown tips (Table, 1). Single step sterilization using of Clorox (10%) for 15 minutes or $HgCl_2$ (0.1%) for 5 minutes each resulted in 22 % clean explants. Increasing the Clorox concentration to 25% and exposure time to 25 minutes increased the percentage of clean explants by about three times (22 to 56%). However, while double step sterilization using pre treatment of the explants with $HgCl_2$ at 0.1% for 5 minutes before the low Clorax (10%) for 15 minutes doubled the percentage of clean explants (22 to 44%), double step sterilization using high Clorox(25%) for 25 minutes followed by either Clorox (10%) for 15 minutes or $HgCl_2$ at 0.1% for 5 minutes on the contrary reduced the percentage of clean explants from 56 to 33%. All of the tips that were treated with ethanol (50 %) for 5 minutes followed by Clorox (10%) for 15 minutes were lost due to contamination. Triple sterilization made of Clorox (25%) for 25 minutes, $HgCl_2$ (0.1%) for 5 minute followed by Clorox (10%) for 15 minutes reduced the success by about three times (56% to 22%).

The uncontaminated and uninjured explants sprouted and proliferated in MS medium irrespective of its BAP enrichments. However the rate of shoot formation was under influence of BAP concentration. The highest shoot formation (7 shoots per explant) was obtained in medium enriched with BAP at 2.25 mg/l and decreased to 4, 3 and 2 shoots as the concentration increased to 3.25, 4.25 and 5.25 mg/l (Figure, 1).

Discussion.

Single step sterilization of crown tip using high Clorox content (25%) and longer exposure time (25 minutes) resulted in higher percentage (56%) of microbial free cultures of crown tip than using low Clorox content (10%) and shorter exposure time (15 minutes) and than using of $HgCl_2$ (0.1%) for 5 minutes which each of them resulted in only 22% microbial free cultures (Table, 1). However, while the effectiveness of single step sterilization using low Clorox (10%) for 15 minutes increased two times (22 to 44%) by double step sterilization in which the explants were first soaked in $HgCl_2$ (0.1%) for 5 minutes before treated with the low Clorox (10%) for 15 minutes, the effectiveness of single step sterilization using high Clorox (25%) for 25 minutes reduced by 41% (56% to 33%) by double step sterilization in which the explants after being treated with high Clorox was subjected again to either low Clorox (10%) for 15 minutes or $HgCl_2$ (1%) for one minute. Pre treating the explants with $HgCl_2$ or ethanol before being treated with high Clorox (25%) was not tested, however, if it were tested it may increase the percentage of microbial free cultures as it did with those explants that were subjected to single step sterilization with low Clorox (10%) and worth being tried. Generally, in case of single and double sterilization, the major cause of unsuccessefull establishment of crown tip was contamination with only few injured ones. However, the major cause of crown tip loss in case of triple sterilization was due to injuries of the tips rather than contamination. This indicate that the concentration or the exposure time

used in the single and double sterilization was below the required and that used in triple sterilization was above the required for obtaining microbial free and uninjured explants. In this study all of the uncontaminated crown tip of pineapple developed into multiple shoots at all of the tested BAP concentrations (Figure, 1). But, at different rate of shoot formation. The problem of Smooth cayenne was the large loss of explant due to contamination. So to develop optimum establishment system for Smooth cayenne pineapple and to minimize the lag time before enough shoots become available for starting commercial propagules production further effort should be focus on sterilization procedure.

Sterilization and establishment are bottleneck phase of any tissue culture system and more effort are needed to develop efficient and reproducible sterilization procdure. Obtaining of uncontaminated explants does not guarantee its establishment. Using of improper hormone concentration could diminish or even block establishment of pineapple explants (Bhatia and Ashwath, 2002; Devi *et al.*, 1997). Establishment of pineapple explants was also effected by type of media (Bordoloi and Sarma,1993), dryness and cultivars (Fitchet, 1990), heat treatment, media states and intactness of buds (Broomes and MacEvan, 1994). The low percentage of successfully sterilized primary explants make investigation and optimization of all possible chemical and physical factors which effect establishment of primary explants a difficult task. Simply most of the explants would be lost due to contamination. Hence it is not a surprise that comprehensive investigation of establishment of primary explants of pineapple as well as other plant was rarely reported. Unless new procedure developed for 100 % clean primary explants, the alternative is series of repeated sterilization and establishment experiments should be tried. Each time, the factors included in the successfully sterilized cultures excluded from the next repeated experiment until all factors and combinations of interest are investigated. In this study we tested only four concentrations of BAP and the experiment had to be repeated several time using new primary explants to compensate for those explants that were lost due to contamination.

References.

Almeida W. A., Santana, B., Rodriguez, G.S., and Costa, M.A., 2002. Optimization of a protocol for the micropropagation of pineapple. *Rev. Brasil. Fruticul*. 24(2): 296-300.

Almeida, W. A. P., Matos, A. P. and Souza, A. D. S. 1997. Effect of benzylaminopurine (BAP) on *in vitro* proliferation of pineapple (*Ananas comosus* (L) Merr.). *Acta, Hort*. 425: 235- 242.

Be L. V. and Debergh, P. C., 2006. Potential low cost micropropagation of pineapple (*Ananas comosus*). *S. Afr. J. Bot.* 72: 191- 194.

Bhatia, P. and Ashwath, N., 2002. Development of a rapid method for micropropagation of a new pineapple (*Ananas comosus* (L) Merr. Clone Yeppoon gold. *Acta Hort*.575: 125- 131

Bordoloi, N. D. and Sarma, C. M., 1993. Effect of various media composition on *in vitro* propagation of *Ananas comosus* (L) Merr. *J. plant Sci Research*. 9: 50- 53.

Broomes, V.F.A. and McEwan, F. A. 1994. Heat treatment for enhanced responsiveness of dormant axillary buds of pineapple. *Turriaba* 44(2): 117- 121

Daquinta, M. A., Cisneros, A. Rodriquez, Y. Escolana, M. Perez. M. C. Luna, I. Borrot, C. G. Martin, P. P. and Hugon, R. 1997. Somatic embryogenesis in pineapple (*Ananas comosus*. L. Merr.). *Acta Hort*. 425: 251- 257.

Das, R. K. and Bhowmik. G. 1997. Some somaclonal variants in pineapple (*Ananas comosus* (L) Merr) plants obtained from different propagation techniques. *Inter. J.Trop.Agric.* 15 (1-4): 95- 100.

Devi Y.S., Mujib, A. and Kundu, S. C. 1997**.** Efficient regeneration potential from long term culture of pineapple. *Phytomorph*. 47(3): 255- 259.

Dewald, M. G, More, G. A. Sherman, W. B. and Evans, M. H. 1988. Production of pineapple plants *in vitro. Plant Cell Report*.7: 535- 537.

Fernando, K., 1986. *In vitro* propagation of Muritus pineapple. *Trop.Agriculturist*. 142: 7-12.

Fitchet, M. 1990. Clonal propagation of Queen and Smooth cayenne pineapples. *Acta. Hort*. 275: 261- 266

Hirimburegama K. and Wijesinghe, L. P. J. 1992. *In vitro* growth of *Ananas comosus* L.Merr (Pineapple) shoot apices on different media. *Acta Hort*. 319:203-208.

Kofi, O. F and Adachi, T. 1993. Effect of cytokinins on the proliferation of multiple shoots of pineapple *in vitro*. *SABRAO Journal*, 1993, 25(1): 59- 69

Liu L. J., Rosa-Marquez, E. and Lazardi, E. 1989. Smooth leaf (spineless) red spanish pineapple (*Ananas comosos* (L) Merr) propagated *in vitro.* *J. Agr. Univ. Puerto Rico* 73: 301- 311.

Murashige T and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue. *Physiol. Plant.* 15: 473- 497

Omokoio, N. D., Fotso, M. A. and Niemenak, N. 2001. Direct *in vitro* regeneration of *Ananas comosus* (L) Merr var cayenne from crown cultivated in liquid medium. *Fruits* 56: 415- 421.

Rahman K. W., Amin, M. N. and Azad, M. A. K. 2001. *In vitro* rapid clonal propagation of pineapple *Ananas comosus* (L) Merr. *Plant Tiss Cult* 11: 47- 53.

Singh, D. B. and Manual, A. B. 2000. Assessment of pineapple plants developed from micropropagation instead of conventional suckering. *Trop. Sci*. 40(4):169-173.

Smith, M. K., Ko, H. L. Hamill, S. D. and Sanewski, G. M. 2002. Pineapple transformation Managing somaclonal variation. *Acta Hort*. 575: 69- 74

Soneji, J. R., Rao, P. S. and Mhatre, M. 2002 Somaclonal variation in micropropagated dormant axillary buds of pineapple (*Ananas-comosus* L., Merr.). *J. Hort. Sci. Biotech*. 77 (1): 28-32.

Sripaoraya S., Marchant, R. Power, J. B. and Davey, M. R. 2003. Plant regeneration by somatic embryogenesis and organogenesis in commercial pineapple (*Ananas comosus* L). *In Vitro Cell. Devl. Biol. Plants*, 39 (5): 450- 454.

Teng, W. L. 1997. An alternative propagation method of *Ananas* through nodule

culture. *Plant Cell Report.* 1997, 16; 454- 457

Wakasa, K. 1989. Pineapple (*Ananas comosus* L. Merr). Biotechnology in Agriculture and Forestry, V. (5), Trees II. pp 13- 29. (Ed. Y.P.S. Bajaj). Springer-Verlang.

Zepeda, C. and Sagawa, Y. 1981. *In-vitro* propagation of pineapple. *Hort. Sci*.16 (4); 495

Table.(1). Effect of sterilization treatments on the percentage of clean Smooth cayenne pineapple crown tips.

Sterilization treatments	Clean sprouting Crown tips (%)
Clorox (25 %) for 25 minutes	56
$HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) for 15 minutes	44
Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute	33
Clorox (10 %) for 15 minutes	22
Clorox (25 %) for 25 minutes; Clorox (10 %) for 15 minutes	33
Clorox (25 %) for 25 minutes; $HgCl_2$ (0.1 %) for 1 minute; Clorox (10 %) for 15 minutes	22
$HgCl_2$ (0.1 %) for 5 minutes	22
Ethanol (50 %) for 5 minutes; Clorox (10 %) for 15 minutes	0.0

Experiment was repeated several times. Each time 5 to 9 crown tips were used per each treatment.

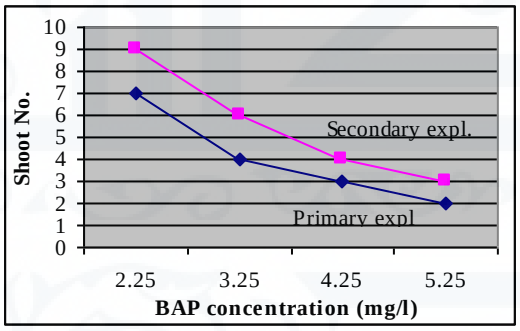

Figure (1) Effect of BAP concentrations on the invitro shoot formation of Smooth cayenne crown tip