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In vitro Growth and Multiplication of Pineapple under Different Duration of Sterilization and Different Concentrations of Benzylaminopurine and Sucrose

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ABSTRACT
The optimal duration of sterilization with 2% sodium hypochlorite as well as the optimal concentrations of Benzylaminopurine (BAP) and sucrose for the *in vitro* culture of MD2 pineapple plants were investigated on MS medium. The research was carried out at the Plant Tissue Culture Laboratory of the University of Cape Coast, Ghana from February to May 2013. The sterilization durations were 10, 15, 20 and 25 min. For the sucrose treatments, 30, 40, 50 and 60 g L^{-1} were used whilst 2, 4, 6 and 8 mg L^{-1} BAP were used as hormonal treatments for bud growth and multiplication, with each treatment replicated 50 times. Initial plant materials were screened for viruses using the PCR method. Sterilizing buds for 25 min. With 2% NaOCl destroyed most of the tender buds and affected regeneration whilst those sterilized for 20 min had above 30% regeneration though there was 10% contamination on the explants. In terms of number of plantlets per explant, 50 g L^{-1} sucrose was found to be better (6.5 plants per plantlet) than those on 60 g L^{-1} sucrose which produced 2.5 plantlets per explant. The superiority of 50 and 30 g L^{-1} sucrose was seen in all the parameters measured. Plants that were cultured on 6 mg L^{-1} BAP had higher mean weights and number of shoots per explant compared to those on 2 and 8 mg L^{-1} BAP.

Key words: *Ananas comosus*, benzylaminopurine, naphthalene acetic acid, sodium hypochlorite

INTRODUCTION

The pineapple (*Ananas comosus*) is one of the major fruit crops of tropical countries. In 2004, world pineapple production was 15,288,018 mt with a yield of 181,303 hectogram/ha (De La Cruz Medina and Garcia, 2005). Fresh pineapple is important on both domestic and export markets. The edible portion of the fruit, which constitutes about 60% of the weight, contains approximately 85% water, 0.4% protein, 15% sugar (sucrose), 0.1% fat and 0.5% fiber (Rangan, 1984; Danso *et al*., 2008). The pineapple fruit is a good source of vitamins A, B and C besides several minerals. Manganese which is found abundantly in pineapple fruit can prevent osteoporosis. Another unusual aspect of pineapple's chemistry is that it has no starch reserves of its own, unlike other fruits.

Pineapple is propagated vegetatively by planting crowns, slips, hapas and suckers. Crowns are the preferred planting material in most pineapple growing countries since they have the potential to develop better root systems. Each crown can be sectioned and planted to increase planting material but this is often not enough. The problem of in adequate supply of healthy planting materials for farmers has been compounded by fusarium wilt. There are also other problems that affect the large scale production of pineapple such as the acquisition of high quality propagules and the slowness of multiplication through the conventional methods (Rugigiero and Koller, 1992).

This has led to the search for alternative ways of producing large numbers of planting materials. Moore *et al*. (1992) developed the first micropropagation protocol which was followed by multiple proliferation techniques by Rangan (1984).

The MD2 pineapple has in recent times replaced the smooth cayenne on the International market due to its superior qualities. It has high Brix with low acidity of 0.4-0.45% (Danso *et al*., 2008). The fruits are good sources of protein digesting enzyme bromelain.

The increased demand for fresh MD2 fruits on both the USA and European markets is therefore expected and to meet the demand of farmers for healthy planting materials, the *in vitro* culture methods needs to be exploited fully. The *in vitro* culture of pineapples for the generation of planting materials and its comparative advantage over the traditional methods in terms of number of plantlets produced and its quality is documented by Danso *et al.* (2008), DeWald *et al.* (1998), Kiss *et al.* (1995), Yapo *et al.* (2011), Be and Debergh (2006), Zuraida *et al.* (2011) and Roostika and Mariska (2003). For the full potential of *in vitro* culture in producing large scale planting materials to be realized, it is essential that the chemical properties of the medium is optimal. Among the factors that contribute to the success of *in vitro* mass production of healthy pineapple plantlets are the addition of Benzylaminopurine (BAP) and Naphtylacetic acid (NAA) to the culture medium. This has been reported by Zuraida *et al.* (2011) that it is possible to produce 6,575 plantlets on a BAP supplemented medium.

The micro propagation protocol has now become fairly standardized for some important crops. The problem with the *in vitro* culture of pineapple especially the MD2 has been with the large percentages of contamination as well as optimizing the sucrose and BAP concentrations in the medium. Though previous study has proven that 2% sodium hypochlorite is the best concentration for sterilizing pineapple bud explants, the duration for effective sterilization to be achieved needs to be established in order not to damage the cells.

The study was therefore carried out to establish the optimum length of time of sterilizing the bud explants of MD2 pineapple in a 2% sodium hypochlorite solution and also determine the optimal concentration of BAP and sucrose for the *in vitro* growth and multiplication of MD2 pineapple.

MATERIALS AND METHODS

The study was conducted at the Plant Tissue Culture Laboratory, Department of Molecular Biology and Biotechnology, University of Cape Coast, between February, 2013 and May, 2013.

Plant material: Healthy MD2 pineapple plants which has been grown in the open field were used as the explant source. The plant materials were screened for viruses using the PCR method by Thomson *et al.* (1996) to ensure that the plant materials used were free of viruses. Buds were extracted from the plant material as described by Roca *et al.* (1978) and Danso *et al.* (2008).

Media preparation: Two types of media for the initiation or regeneration and the multiplication of buds were prepared. MS medium (Murashige and Skoog, 1962) supplemented with 1×10^{-5}, 1×10^{-3} and 30 g L^{-1} of benzylaminopurine, napthalene acetic acid and sucrose, respectively was used as the initiation medium. The pH of the medium was adjusted to 5.8 with NaOH and HCl and agar at 6 g L^{-1} was added as a solidifying agent. The medium was then dispensed into culture vessels (test tube), labeled and autoclaved at 121°C at 1.5 kg cm^{-1} (15 psi) pressures for 15 min.

Isolation and sterilization of explants: Pineapple suckers of the MD2 cultivar were collected from the field courtesy Mr. Aaron Asare of the Molecular Biology and Biotechnology Department.

Explants were isolated from initial mother plants in the culture room, by carefully removing the leaves on the suckers to expose the buds on the stem. The buds were carefully isolated into a bowl and randomly divided into four groups. The buds were washed with liquid soap to get rid of the dirt. They were then rinsed with lot of water to get rid of the soapy solution on the buds. The buds were sterilized with 2% sodium hypochlorite for different time durations; 10, 15, 20 and 25 min representing the various treatments with each of them having 50 bud explants. The 2% sodium hypochlorite had been established as the optimal concentration for sterilization. After sterilization, the buds were trimmed to remove the dead surfaces. They were then inoculated on the initiation medium described above, in test tubes. The test tubes were sealed with cotton wool and then wrapped with parafilm. The cultures were incubated at 25±2°C, under 16 h light and an intensity of 2500 Lux. The experiment was observed daily for growth. Bud explants were cultured for 8 weeks and data was taken on percentage regeneration from the various treatments.

Effect of different concentrations of BAP and sucrose on the growth and multiplication of pineapple *in vitro:* A second medium for the multiplication of the regenerated buds was supplemented with different concentrations of BAP (2, 4, 6 and 8 mg L^{-1}) with 30 g L^{-1} of laboratory grade sucrose as the carbon source and solidified with 6 g L^{-1} agar. The 30 g L^{-1} sucrose concentration used was from previous experiments for the preparation of MS medium. The pH of the medium was adjusted to 5.8 with drop wise of 0.5 N NaOH and HCl. The medium was then dispensed into the culture vessels (test tubes) labelled and autoclaved at 121°C at 1.5 kg cm^{-1} (15 psi) pressures for 15 min. Eight weeks old explants from the initiation medium were then cultured on the different media with varied BAP concentrations with each treatment having 50 explants as replications.

Data was taken at 8, 10, 12, 14 and 16 weeks of culturing on and number of buds per plantlet and weight of plantlet (Fig. 1). Another MS medium was supplemented with different concentrations of sucrose (30, 40, 50 and 60 g L^{-1}), 6 mg L^{-1} BAP (established from previous experiment) and solidified with 6 g L^{-1} agar, to investigate their effects on the growth and multiplication of MD2 pineapple. Sterile cultures from the initiated buds were used and cultured in the test tubes containing media of different sucrose concentrations with each

Fig. 1(a-b): (a) Pineapple stem with buds and (b) Isolated buds

treatment having 50 explants. The test tubes were sealed with cotton wool and then wrapped with parafilm. They were then incubated at 25±2°C, under 16 h of light and an intensity of 2500 Lux.

Data was taken at 8, 10, 12, 14 and 16 weeks of culturing on number of leaves, plantlet height, number of shoots per plantlet, number of roots per plantlet and shoot weight.

Data collection: Data was collected over 16 weeks of culture on the following: Number of roots, plant height, number of leaves, number of shoots and weight of plantlets. The leaf lengths were measured from the leaf base to the tip of the leaf.

Statistical analysis: Collected data was subjected to analysis of variance (ANOVA), using statistical package Minitab 15.

RESULTS

The level of contamination in the regeneration medium was inversely proportional to the duration of sterilization. At 25 min. of sterilization, the level of non-contamination was 100% whilst 10 min sterilization gave 55% non contamination Fig. 2. Similarly, sterilization duration affected the percentage regeneration of the explants. When explants were exposed to the sterilant for 10, 15 and 20 min, regeneration was above 30% and for 25 min of sterilization, it was 25%.

The average number of plantlets per explant varied with the different concentration of sucrose used in the MS medium.

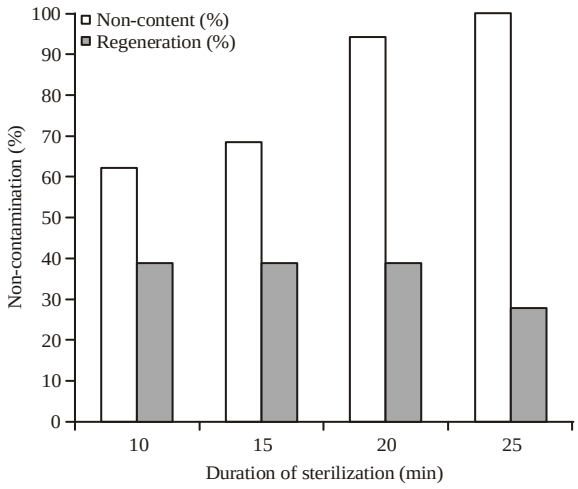

Fig. 2: Effect of duration of 2% sodium hypochlorite sterilization on percentage of contamination and regeneration

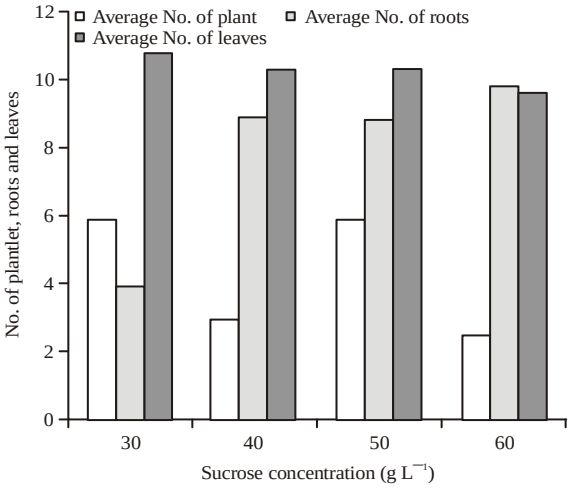

Fig. 3: Effect of different concentrations of sucrose on growth of pineapple plantlets *in vitro*

Buds which were cultured on media supplemented with 30 and 50 g L^{-1} sucrose gave 6 and 6.5 plantlets per explants while media supplemented with 40 and 60 g L^{-1} sucrose gave 3 and 2.5 plantlets per explant, respectively (Fig. 3). The difference between the number of plantlets on media supplemented with 30 and 50 g L^{-1} sucrose and those supplemented with 40 and 60 g L^{-1} sucrose were significant. The mean number of roots per plantlet was positively linked to the sucrose concentration. Plantlets on 30 g L^{-1} had 4 roots per plantlet and those on 60 g L^{-1} had 10 roots per plantlet. Number of leaves however decreased slightly with increase in sucrose concentration from 11 leaves for those on 30 g L^{-1} to 10 leaves for those cultured on 60 g L^{-1} sucrose (Fig. 3).

The weight of shoots varied with the sucrose concentration in the medium. Shoots on medium with 30 g L^{-1} had the highest weight of 0.8 g per plantlet while those

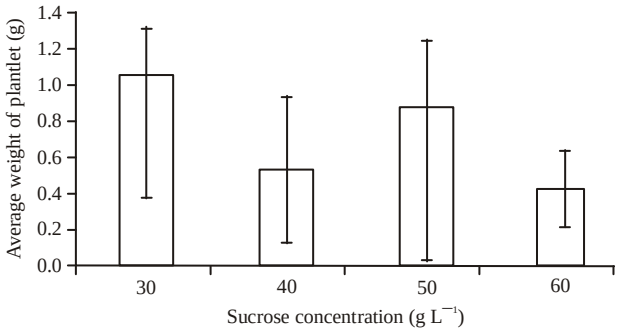

Fig. 4: Effect of different concentrations of sucrose on the weight of pineapple plantlets *in vitro*

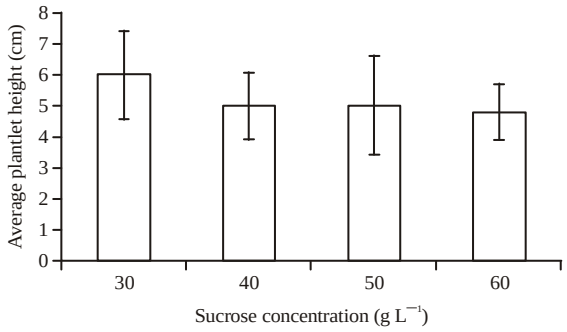

Fig. 5: Effect of different concentrations of sucrose on plantlet height

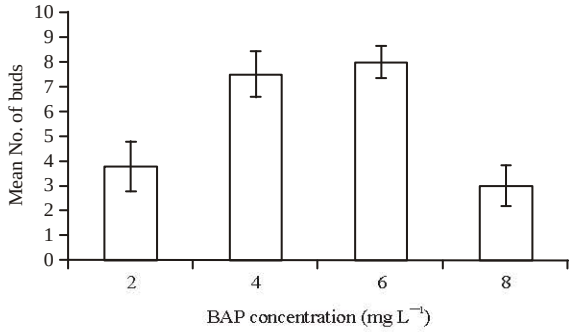

Fig. 6: Effect of different concentration of BAP on shoot number

on 60 g L^{-1} sucrose has a shoot weight of 0.3 g, showing a significant difference between 30 and 60 g L^{-1} sucrose (Fig. 4).

Plantlets that were cultured on 30 g L^{-1} sucrose grew significantly taller than those cultured on the other media, supplemented with 40, 50 and 60 g L^{-1} sucrose after 16 weeks of culture. An average height of 6 cm was recorded for plantlets that were cultured on 30 g L^{-1} whilst the rest all recorded a height of 5 cm (Fig. 5).

The number of buds per explant also varied with different concentrations of BAP in the medium. Average of 7.5 and 9 buds per explant were produced from explants cultured on media supplemented with 4 and 6 mg L^{-1} BAP, respectively which was significantly different from those cultured on media

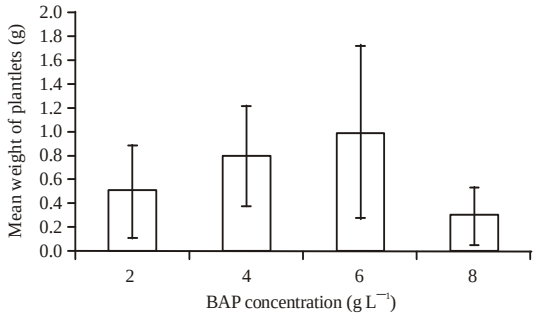

Fig. 7: Effect of different concentration of BAP on mean weight of plantlet

supplemented with 2 and 8 mg L^{-1} BAP with 4 and 3.5 buds per plantlet respectively (Fig. 6). Lower 2 mg L^{-1} or higher concentrations 8 mg L^{-1} in the medium produced less number of plantlets.

Shoot weight tended to increase with increase in the concentration of BAP from 2-6 mg L^{-1} and declined beyond a concentration of 6 mg L^{-1} BAP.

The lowest 2 mg L^{-1} and the highest 8 mg L^{-1} BAP supplemented media produced shoots with the least weight of 0.4 and 0.2 g/shoot. Whilst shoots cultured on 4 and 6 mg L^{-1} had weights of 0.8 and 1 g, respectively as shown in Fig. 7.

DISCUSSION

The success of *in vitro* culture of plants depends to a large extent on the effectiveness of the process of sterilizing the explants. Various researchers have used different chemicals to sterilize explants (Teixeira *et al*., 2006; Usman *et al*., 2012). There have been instances where different researchers have used the same chemical of the same concentration but the duration of sterilization has been different, even for very similar plant materials. Sodium hypochlorite (NaOCl) has largely been the sterilant most researchers have used but at different concentrations and duration of sterilization. In present study, 2% NaOCl was used which had been established from previous unpublished experiment. What is being investigated here is the different sterilization durations to establish the right duration for sterilization. It was observed that percentage contamination decreased with increased duration of sterilization. At 25 min no contamination was observed but regeneration of explants into plantlets was low compared to durations of 10, 15 and 20 min where 45, 40 and 20% contamination respectively was observed but regeneration of plantlets was better than those sterilized for 25 min. Khan *et al*. (2004), Ibrahim *et al*. (2013) and Rodrigues *et al*. (2013) have all reported on the negative effect of prolonged sterilization of pineapple tissues in NaOCl. Rodrigues *et al*. (2013) used concentrations of 5, 10, 15 and 20% for 15 min and reported 5% as the best concentration. The low level of regeneration with prolonged sterilization could be due to the effect of the active ingredient in NaOCl on

the delicate tissues which could lead to the killing of the actively growing parts of the explants. This thought has also been confirmed by Teixeira *et al.* (2006).

The positive effect of BAP on shoot formation in the *in vitro* culture of pineapple has also been reported by Khan *et al.* (2004), Danso *et al.* (2008), Zuraida *et al.* (2011), Usman *et al.* (2013) and Farahani (2014). However, in a few cases, as observed by Ibrahim *et al.* (2013), shoot formation did not occur at the various levels of BAP used for the *in vitro* culture of pineapples but rather observed the formation of callus.

The influence of BAP on the cellular division and shoots growth induction to the axillary buds has been the underlining factor for shoot multiplication of plants *in vitro*. BAP is known to stimulate cell division and axillary bud proliferation (Kyte and Kleyn, 1996). The contribution of BAP to the regeneration of plantlets from shoot apices of pineapple has also been reported by Firoozabady and Gutterson (2003). It was observed in present study that explants cultured on medium supplemented with 6 mg L^{-1} BAP gave the highest number of shoots per explant as well as the highest mean weight and plant height. Number and weight of shoots increased as BAP concentration increased from 2-6 mg L^{-1} and decreased significantly on medium supplemented with 8 mg L^{-1}. Thus higher BAP concentration tended to negatively affect shoot number and weight. Similarly, many researchers such as Sripaoraya *et al.* (2003) and Hamad and Taha (2008) have reported on the effect of higher BAP concentration in a medium on plant growth. At higher concentration of BAP, the exogenous hormone competes with the endogenous BAP for the functional sites therefore affecting the overall effect (Nielsen *et al.*, 1995). Even though 6 mg L^{-1} BAP was the best in the present study for shoot growth, some researchers have found lower or higher concentrations than 6 mg L^{-1} to be the best for shoot growth. Be and Debergh (2006), Zuraida *et al.* (2011) and Farahani (2014) contrary to the present results, have reported variously that 5 and 1.5 mg L^{-1} BAP gave the best shooting *in vitro*. Again Danso *et al.* (2008) revealed that 7.5 mg L^{-1} gave the best proliferation of MD2 pineapple. In present study, different culture conditions were used. For example, light intensity and photoperiod compared to the other researchers. This could account for the difference between their optimal BAP concentration and ours. For example Danso *et al.* (2008) cultured the explants under light intensity of 2800 Lux and also combined BAP and NAA for shoot growth whilst we used BAP and NAA for bud initiation but only BAP alone was used for shoot growth and multiplication all under light intensity of 2500 Lux.

Sucrose serves as the energy source for cell multiplication and growth of plants *in vitro*. It helps in the production of protein, total enzyme activity and specific as enzyme activity as reported by Ngampanya and Phongtongpasuk (2006). It also provides the energy necessary for secondary metabolites production in plants leading to an increase in biomass (Zhong and Yoshida, 1995; Perez *et al.*, 2003). The effect of various concentration of sucrose in the medium on plantlet multiplication, weight, number of leaves, number of roots and plant height was investigated. Plant height was better on 30 g L^{-1} sucrose compared to the other treatments. Number of roots and number of leaves appeared to be very similar in all the treatments. Media supplemented with 30 and 50 g L^{-1} sucrose gave more shoots compared to those cultured on 40 and 60 g L^{-1} sucrose. This was contrary to the finding of Ngampanya and Phongtongpasuk (2006) who had an increase in shoot number as sucrose concentration was increased from 30-45 g L^{-1} which is also collaborated by Abd El Gawad *et al.* (2010) the fewer number of shoots on medium with 60 g L^{-1} sucrose was expected. Medium water potential has been reported by Buah *et al.* (2011) to affect the growth of plantlets *in vitro*. Even though they reported on how increased level of gelling agent can lower the medium water potential, it is believed that high sucrose level in a tissue culture medium could also lower medium water potential due to its effect of osmosis and nutrient uptake. This will consequently affect multiplication of plants *in vitro*.

CONCLUSION

NaOCl at 2% is effective for the sterilization of pineapple explants when the plant material is sterilized for 20 min beyond which the cells die to affect renegeneration. For better growth and multiplication of pineapple plantlets *in vitro*, BAP at 6 mg L^{-1} and sucrose at 30 mg L^{-1} is optimum.

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