



Effect of sucrose, pH and medium states on *in vitro* shoot formation and growth of Moris pineapple (*Ananas comosus* L. Merr.)

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Abstract: The effect of medium states (solid, semi solid and liquid) of full strength MS medium enriched with 6-benzylaminopurine (BAP) at 2.0 mg/l on *in vitro* shoots formation and shoot length of Moris pineapple were tested at 16 combinations of sucrose (10, 20, 30 and 40 g/l) and pH (5.0, 5.7, 6.0 and 6.5). The highest shoot formations (7 shoots/ explant) were obtained in liquid and solid media each adjusted to pH 5.0 but enriched with different sucrose concentrations, sucrose at 20 g/l for liquid and at 30 g/l for solid medium. Increasing the medium sucrose to 40 g/l or adjusting the medium to pH 6.0 caused 50 % decline in the shoot formation capacity in both medium states. However, while that decline could be reversed in liquid medium by adjusting the pH to 6.5, such pH adjustment failed to overcome the inhibitory effect of the sucrose at 40 g/l in the solid medium. Out of 16 combinations of sucrose and pH, liquid medium (no agar added) was better than solid (7.0 grams of agar /l) and semi solid (3.5 grams of agar /l) at 8 combinations, equal to solid at 4 and to semi solid at 5 combinations and less than solid at 3 and than semi solid at 2 combinations. Adopting of the commonly used combination of sucrose at 30 g/l and pH 5.7 not only did not fit all medium states but also resulted in lower shoot formation (4 shoots) than the possibly obtainable (7 shoots). Simple modification of the medium pH (pH 5.0 instead of 5.7) doubled the rate of shoot formation.

Key words: Sucrose concentration, pH; Pineapple, *Ananas comosus*, Media states.

INTRODUCTION

Solid MS medium was recommended by several researchers for pineapple *in vitro* culture and proven to have high potential for production of thousands of propagules (Sripaoraya *et al.*, 2003, Hamad and Taha 2008, Pérez *et al.*, 2009). At the same time, pineapple shoot on semi liquid (Akin-Idowu *et al.*, 2014), static (Almeida *et al.*, 2002, Pérez *et al.*, 2012) and agitated liquid cultures (Soneji *et al.*, 2002a), filter paper bridge (Mathews and Rangan 1979, Fernando 1986) and temporary immersion system (Escalona *et al.*, 1999, Firoozabady and Gutterson 2003) were found to be much better than solid

medium for *in vitro* multiplication of pineapple. However, these comparisons were made at fixed sucrose (30 g/l) and pH (5.7) in both medium states. For multiplication on solid MT medium, (Fitchet 1990) adjusted the pH to 5.0 while (Teixeira *et al.*, 2006) used pH 6.5 during multiplication in liquid MS medium. At fixed sucrose (20 g/l), comparison of different pH adjustments during multiplication stage showed that liquid was better than solid medium and adjusting to pH 5.0 was better than adjusting to pH 5.7, 6.0 and 6.5 (Hamad, 2017a). Sucrose at 20 (Soneji *et al.*, 2002b, a) Smith, *et al.*, 2002.), 35 (Kofi and Adachi 1993) and 40 g/l (Almeida, *etal*, 1997) were used for establishment and multiplication.

(Sripaoraya *et al.*, 2003) used sucrose at 50 g/l during establishment but decreased the sucrose to 30 g/l during multiplication stage. Comparisons of sucrose effect on pineapple culture were made at range of 10, 20, 30 and 40 g/l for shoot formation (Hamad, 2017b), callus induction (Benega, *et al.*, 1997) and at range of 0.0, 30, 60, 90 and 120 g/l for total fresh weight per bioreactor (Pérez *et al.*, 2004). Highest shoot formation and highest fresh weight were obtained at 30 g/l while either sucrose levels were equally effective for callus induction. Since sucrose is an indispensable component and both of sucrose and agar comprise the largest part of the medium components, determination of optimum level is not only important for *in vitro* shoot formation and growth but also as a cost factor. Application of extra amount above that required for the optimal shoot formation is just an avoidable added cost. The objective of this study is to compare the effect of combinations of four concentrations of sucrose (10, 20, 30 and 40 g/l) and four pH adjustments (5.0, 5.7, 6.0 and 6.5) on shoot formation and growth of Moris pineapple on three states (solid, semi solid and liquid) of full strength MS medium enriched with BAP at 2.0 g/l.

MATERIALS AND METHODS

Full strength MS medium were prepared from stock solutions and enriched with BAP at 2.0 mg/l. The medium was divided into 4 beakers (750 ml each) marked A, B, C and D and 7.5, 15.0, 22.5 and 30.0 grams of sucrose were added to each beaker respectively to give a sucrose enrichment of 10, 20, 30 and 40 g/l. The content of each beaker was divided into another 4 beakers marked with same marks (A, B, C and D) plus numbers from 1 to 4 and the medium pH of the beakers marked with 1, 2, 3 and 4 was adjusted to pH 5.0, 5.7, 6.0 and 6.5 respectively. The content of each beaker of the same sucrose and pH combination was divided into 9 glass jars (20 x 5 cm) and each three jars marked with the same marks on the beaker plus S, E and L. Agar at 0.14

and 0.7 grams was respectively added to each jar marked with S and E letter to give medium solidification of 7.0 and 3.5 g/l and no agar was added to jars with L letter. The jars were closed by autoclavable plastic lids and the medium was autoclaved at 121 °C and 1.5 kg / cm² for 25 minutes and kept in a culture room. One shoot from Moris stock cultures was cultured per each jar under laminar cabinet and the cultures were incubated under constant temperature of 25 °C and 16 hours of light provided by cool white fluorescent lamps. After two months of incubation, the multiple shoot buds complex of each culture were picked out of the jars and separated into individual shoots for counting the shoots and measuring their length. Each jar was considered as a replicate and the data were subjected to ANOVA analysis and means separation by Duncan Multiple Range Test at $p \leq 0.05$ using SPSS statistical package No. 11.

RESULTS

Analysis of variance (Table, 1) showed that the shoot formation and the shoot length were under direct effect of medium states ($p \leq 0.0166$ and $p \leq 0.0001$ respectively) and sucrose concentrations ($p \leq 0.0002$ and ≤ 0.00003 respectively). The sucrose effect on shoot formation and shoot length was influenced by a significant interaction with pH ($p \leq 0.0039$ and ≤ 0.00001). On the contrary, the medium states effect on shoot formation was independent of pH ($p \leq 0.3936$) and sucrose ($p \leq 0.0764$) while the medium state effect on shoot length was influenced by the medium sucrose content ($p \leq 0.0141$) but independent of pH ($p \leq 0.0764$). Medium pH on the other hand had no direct independent effect on both of shoot formation ($p 0.3031$) and shoot length ($p \leq 0.7794$), but influenced both of shoot formation ($p \leq 0.0039$) and shoot length ($p \leq 0.00001$) via interaction with sucrose content of the medium. Furthermore, the three factors together showed no significant collective interaction on shoot formation ($p \leq 0.1520$) and shoot length ($p \leq 0.2460$). The highest shoot formation (7 shoots) were obtained in liquid medium enriched with sucrose at 20 g/l and also in solid medium enriched with sucrose at 30 g/l both adjusted to

pH 5.0. The lowest shoot formation (3 shoots per explant) as well as equal shoot formation (6, 5 and 4 shoots) was also obtained in liquid and solid medium, but at different combinations of sucrose and pH (Table, 2). At fixed pH of 5.0, equal shoot formation (3 shoots) were obtained in both solid and liquid media enriched with sucrose at 10 and 40 g/l while in media enriched with sucrose at 20 g/l, liquid produced more shoots (7 shoots) than solid (5 shoots) medium. In media enriched with sucrose at 30 g/l, solid on the contrary produced more shoots (7 shoots) than liquid (5 shoots). At fixed pH of 5.7, solid medium enriched with sucrose at 10 and 40 g/l produced more shoots (4 and 5 shoots) than liquid medium (3 and 4 shoots) while in media enriched with sucrose at 20 and 30 g/l, liquid medium on the contrary produced more (6 and 5 shoots respectively) shoots than solid medium (3 and 4 shoots). At fixed pH of 6.0, equal shoot formation (4 shoots) were obtained in solid and liquid media enriched with sucrose at 10 and 20 g/l (low sucrose enrichment) while in media enriched with sucrose at 30 and 40 g/l (high enrichment) liquid medium produced more (6 and 6 shoots) shoots than solid medium (5 and 3 shoots). At pH 6.5, liquid medium enriched with sucrose at 20, 30 and 40 g/l produced more (6, 5 and 6 shoots respectively) shoots than solid medium (3, 4 and 3 shoots) and in media enriched with sucrose at 10 g/l equal shoot formation (4 shoots) were obtained in both medium states.

Table (1). Main and interaction effect of medium states, sucrose and pH on *in vitro* shoot formation and shoot length of Moris pineapple

Factors	Df	Parameters	
		Shoot/ explant.	Shoot length(mm)
		p values	
Medium states	2	0.0166 *	0.0001 **
Sucrose	3	0.0002 **	3.9E-08 **
pH	3	0.3031	0.7994
States*Sucrose	6	0.0764	0.0141 *
States*pH	6	0.3935	0.6044
Sucrose*pH	9	0.0039 **	1.0E-05 **
States*Sucrose*pH	18	0.1520	0.2460
Error	96		
Total	144		

DISCUSSION

Previous studies of pineapple tissue culture concluded that using of semi liquid(Akin-Idowu *et al.*, 2014), static liquid(Almeida *et al.*, 2002, Pérez *et al.*, 2012), agitated liquid cultures (Soneji *et al.*, 2002a) and filter paper bridge (Mathews and Rangan 1979, Fernando 1986) resulted in higher shoot formation than solid medium. This study demonstrated that superiority of the medium state depended on the sucrose enrichments

Table (2). Effect of medium states, sucrose and pH on *in vitro* shoot formation of Moris pineapple

Sucrose (g/l)	pH	Medium states		
		Solid	Semi	Liquid
Shoots per explant				
10	5.0	3 c	3 c	3 c
	5.5	4 bc	3 c	3 c
	6.0	4 bc	3 c	4 bc
	6.5	4 bc	4 bc	4 bc
20	5.0	6 ab	5 abc	7a
	5.5	3 c	3 c	6 ab
	6.0	4 bc	4 bc	4 bc
	6.5	3 c	4 bc	6 ab
30	5.0	7a	5 abc	5 abc
	5.5	4 bc	4 bc	5 abc
	6.0	5 abc	4 bc	6 ab
	6.5	4 bc	4 bc	5 abc
40	5.0	3 c	5 abc	3 c
	5.5	5 abc	6 ab	4 bc
	6.5	3 c	3 c	6 ab
	6.0	3 c	3 c	4 bc

Data were means of 3 explants cultured on full strength MS medium enriched with BAP at 2.0 mg/l and incubated for 60 days
Means of the same parameters (shoot per explant and shoot length) followed by the same letter were not significantly different at $p \leq 0.05$ according to Duncan Multiple Range Test.

and pH adjustments and the solid medium had a very critical requirement of narrow range while liquid medium had a wider range of sucrose-pH combinations (Table, 2). The highest shoot formation (7 shoots) was obtained in solid as well as in liquid medium enriched with sucrose at 30 and 20 g/l respectively and adjusted to pH 5.0.

The lowest shoot formations (3 shoots) was obtained also in liquid and solid medium enriched with sucrose at 10 and 40 g/l and adjusted to pH 5.0. In both of solid and liquid medium the shoot formation ranged from a minimum of 3 to a maximum of 7 shoots. Furthermore, in 8 out of 16 combinations of sucrose and pH, liquid medium resulted in more shoots than both solid and semi solid media (50 % of the cases).

At other 5 combinations (31 %), the shoot formation in liquid was equal to that in solid and at other 3 combinations (19 %) was less than that of solid. At 5 combinations (31 %), the shoot formation in liquid medium was equal and at 2 combinations (13 %) was less than that in semi solid medium. Solid medium was better than liquid in 3 combinations (19 %) and than semi solid at 5 combinations (31 %) and less than semi solid at 3 (19 %) of the sucrose-pH combinations. Hence, any of the medium states could be claimed better than the other depending on which sucrose-pH combination was used. However, being only 25 % of the sucrose-pH combinations (4 of 16) in solid and semi solid media resulted in more than 5 shoots (71 % of the possibly obtainable shoots (7 shoots) while 50 % of the combinations (8 of 16) in liquid medium resulted in more than 5 shoots (71 % of the possibly obtainable shoots (7 shoots) indicated that selection of proper sucrose and pH is critical and very specific in solid than in liquid medium. Generally, liquid state with low sucrose enrichment would be favored for low cost. This study demonstrated that sucrose at 30 g/l and pH at 5.7, which is the most common used combination for *in vitro* culture, is not proper for Moris pineapple. Table 2 showed that in solid medium keeping sucrose at 30 g/l and decreasing the pH to 5.0 and lowering both (sucrose to 20 g/l and pH to 5.0) resulted in 100 % increase in shoot formation. Similar, in liquid medium keeping the sucrose at 30 g/l and increasing the pH to 6.0, keeping pH at 5.7 and decreasing sucrose to 20 g/l, increasing both (the sucrose to 40 g/l and pH to 6.5) and lowering both (sucrose to 20 g/l and pH to 5.0) resulted in 17 to 20 % increase in shoot formation. Using solid medium adjusted to

pH 5.7, Hamad (2017b) found that proper sucrose concentration varied among different pineapple cultivars. (Sucrose at 30 g/l for Moris and at 20 g/l for Smooth cayenne). However, at fixed sucrose enrichment (20 g/l), Moris cultured in liquid medium adjusted to pH 5.0 produced more shoots than in solid medium (Hamad, 2017a). It is important to point out that pH adjustment was a simple none cost item and generally ignored factor that could doubled or drastically reduce the shoot formation (Table, 2).

Liquid medium enriched with sucrose at 10 g/l and adjusted to pH 5.0 resulted in low shoot formation but the rate increased when medium pH increased while when enriched with sucrose at 20 g/l resulted in higher shoot formation but the rate decreased when the medium pH increased. If this trend of response to pH adjustments is persisted, using of pH range higher than 6.5 in medium enriched with sucrose at 10 g/l and pH range lower than 5.0 in medium enriched with sucrose at 20 g/l may improve the shoot formation and is suggested for future testing. The different rate of shoot formation at different combinations of sucrose enrichment and pH adjustment in same medium state and to the same combination in different medium states indicated that adopting of one single sucrose-pH treatment as it is commonly done could not lead to a valid comparison of medium states and investigation of the physiology of shoot formation. In fact, one fixed combination of sucrose and pH could drastically reduce the shoot formation in one medium state while doubling the shoot formation in the other one (Table, 2). Medium states, sucrose contents and pH adjustments played important role in the process of shoot formation. However, that role is not clearly understood yet.

It is generally assumed that the promotion effect of liquid medium is due to the accessibility of medium component to the explants. Liquid state was in general better than solid and sucrose at 40 g/l inhibited the shoot formation (Table, 2). However, the superiority of liquid state and the inhibition of high sucrose content could be blocked and even reversed by pH adjustment.

Liquid medium enriched with sucrose at 20 g/l at all pH except 6.0 and with sucrose at 30 g/l at all pH except pH 5.0 resulted in more shoots than solid medium. Adjusting the medium enriched with sucrose at 20 g/l to pH 6.0 blocked the promotion effect of liquid medium (equal shoots obtained in both medium states) while adjusting the medium enriched with 30 g/l to pH 5.0 reversed the situation and solid promoted more shoots than liquid (Table, 2). That is the pH and sucrose affected the ability of liquid state to promote shoot formation. If the superiority of liquid medium was due to nutrient accessibility, the statistical analysis (Table, 1) should have shown that the medium states had significant interaction with the other factors that reverse the state superiority. Table 1 showed that medium states affect was independent of the sucrose concentrations and pH adjustments. Neither the interaction of medium states with pH and with sucrose each alone nor the collective interaction of the three factors together was significant while a significant interaction was detected between sucrose and pH.

This indicated that the shoot formation was controlled by the interaction of pH and sucrose more than the medium state. The interaction of sucrose and pH presumably resulted in either fast or slow uptake of sucrose or formation of complex that could either promote or inhibit the shoot formation depending on how much sucrose the medium contained and to what pH was adjusted rather than the types of medium state. In conclusion, this study demonstrated that *in vitro* shoot formation is controlled by the sucrose and pH of the medium more than the medium state. The medium pH adjustment which is none cost item and generally ignored factor is essential for obtaining a substantial increase in shoot formation. The contradicting responses to different sucrose-pH combinations in same and different medium states could help in the selection of specific combinations of sucrose concentrations and pH adjustments that would be proper for investigation of the physiology of *in vitro* shoot formation, elucidation of pH and sucrose roles and developing of medium for optimum shoot formation and elongation.

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تأثير حالة الوسط ودرجة حموضته ومحتواه من السكروز علي عدد النموات المتكونة من عزلة نبات أناناس صنف موريس

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المستخلص: اختبر تأثير ثلاث حالات (الحالة السائلة والصلبة والنصف صلبة) من وسط موراشيغ و سكوك (MS) المحتوي على هرمون 6-benzylaminopurine (BAP) بتركيز 2 ملجرام في اللتر، والمعدل محتواه من السكروز ودرجة حموضة الوسط (pH) ب 16 توليفة مكونة من أربعة تركيزات من السكروز (10 ، 20 ، 30 ، و 40 جرام في اللتر) وأربع درجات من الحموضة pH (5.0، 5.7، 6.0 و 6.5) على عدد النموات المنتجة من عزلة نبات أناناس صنف موريس . استعمل الوسط السائل المحتوي على سكروز بتركيز 20 جرام في اللتر والوسط الصلب المحتوي على سكروز بتركيز 30 جرام في اللتر وكل منهما حموضته (pH) 5.0 وهذا أدى إلى الحصول على أعلى متوسط عدد نموات من العزلة (7 نموات)، زيادة تركيز السكروز إلى 40 جرام في اللتر أو تعديل درجة حموضة الوسط (pH) إلى 6.0 أدى إلى أن تفقد العزلة 50 % من قدرتها على إعطاء نموات في كل من الوسطين السائل والصلب . في حين كان من الممكن التغلب على فقدان القدرة على إعطاء النموات وعكسه في حالة الوسط السائل بواسطة تعديل حموضة الوسط إلى 6.5 مثل هذا التعديل فشل في التغلب على التأثير المثبط للتركيز المرتفع للسكروز (40جرام) في حالة الوسط الصلب . في العموم فإن استعمال الوسط السائل أعطى أكثر عدد نموات من تلك المتحصل عليها من استعمال الوسط الصلب والوسط النصف الصلب في ثماني توليفات وعدد نموات متساوية مع الوسط الصلب في 4 توليفات، ومتساوية مع الوسط نصف الصلب في 5 توليفات وعدد نموات أقل من الوسط الصلب في أربعة توليفات، وأقل من نصف الصلب في توليفتين من إجمالي 16 توليفة من السكروز و درجة الحموضة، استعمال التوليفة الأكثر شيوعاً واستعمالاً في دراسات زراعة الأنسجة (سكروز 30 جرام في اللتر ودرجة حموضة 5.7) لم تكن فقط غير ملائمة لكل حالة من حالات الوسط الثلاثة بل أدت إلى إعطاء عدد أقل من النموات (4 نموات) مما هو بمقدور العزلة أن تعطيه (7 نموات) لو استعملت توليفة مثلى من تركيز السكروز ودرجة حموضة الوسط ، النتائج أظهرت أن إجراء تغيير بسيط في الوسط وذلك باستعمال درجة حموضة (pH) 5.0 بدلاً من درجة الحموضة الشائعة الاستعمال (5.7) أدى إلى مضاعفة عدد النموات التي يمكن الحصول عليها من العزلة الواحدة .

الكلمات المفتاحية: تركيز السكروز ، درجة الحموضة ، الأناناس ، حالة الوسط.