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Effect of Hormone Types and Concentrations on *In Vitro* Multiplication and Growth of Pineapple (*Ananas Comosus* (L.) Merr.) Cv Moris on Liquid MS Medium

Abdelhamid M. Hamad
Horticulture Department, Faculty of Agriculture, Omar Al-Mukhtar University, Libya

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Abstract: The effect of two cytokinins, 6-benzyleaminopurine (BAP) and kinetin (KN) and two auxins, naphthalene acetic acid (NAA) and indole acetic acid (IAA) each applied at 10 concentrations with an increment of 0.5 mg/l in the *in vitro* shoot formation of Moris pineapple was evaluated in static liquid MS medium. The highest shoot formation (9 shoots per explant) was obtained in medium enriched with NAA but at high concentration (4.5 mg/l). The second best was 8 shoots per explant obtained in medium enriched with BAP at 2.0 mg/l. Out of 10 concentrations, NAA was better than BAP at the highest 5 and less at lowest 5 concentrations and at all concentrations both hormones resulted in production of more shoots per explant than hormone free medium. Except at two concentrations, NAA and BAP were better than KN. Either one of these three hormones, BAP, NAA, KN could be claimed, depending on the tested range of concentrations, better than the others for *in vitro* shoot formation of Moris pineapple. All of the IAA concentrations, on the other hand, failed to produce more shoots than hormone free medium. But, the longest shoot (56 mm) obtained in medium enriched with IAA at 2.5 mg/l and the shortest shoots (6 mm) on medium enriched with KN at 4.0 mg/l. Seven of the IAA concentrations resulted in shoots longer than hormone free medium (27 mm) while the shoots length on the BAP, NAA and KN contained media was two times shorter than hormone free medium. BAP at 2.0 mg/l was the best treatment for multiplication and IAA at 2.5 mg/l was the best for elongation of Moris pineapple.

Key words: Pineapple, *Ananas comosus*, liquid culture, *in vitro* multiplication.

INTRODUCTION

Tissue culture of pineapple were reported using solid medium(Bhatia and Ashwath 2002, Sripaoraya *et al.,* 2003, Hamad and Taha 2008a, Hamad and Taha 2008c, b, Dutta *et al.,* 2013, Nelson *et al.,* 2015) Hamad,2017), semi liquid (Akin-Idowu *et al.,* 2014), double phase system (Pérez *et al.,* 2012), filter paper bridge (Mathews and Rangan 1979, 1981), direct placement in a stationary liquid (Almeida *et al.,* 2002, Hamad and Taha 2003, Be and Debergh 2006, Teixeira *et al.,* 2006, Pérez *et al.,* 2012) and agitated liquid culture at 50 (Kofi and Adachi 1993), 70(Fernando 1986), 100 (Aydieh *et al.,* 2000, Soneji *et al.,* 2002, Zuraida *et al.,* 2011) and 120 rpm (Zepeda and Sagawa, 1981). In cases in which medium states effect was compared, liquid was better than solid medium and agitated culture was better than the stationary one. Bioreactor system was also used to reduce the cost of pineapple *in vitro* culture (Escalona, *et al*., 1999; Firoozabady and Gutterson, 2003; Scherer, *et al*., 2013). The most commonly used hormone for multiplication was BAP. It was used singly (Zepeda and Sagawa, 1981; Fernando, 1986; Aydieh *et al*., 2000; Almeida

Corresponding Author: Abdelhamid M. Hamad abdelhamidhamad@gmail.com, Faculty of Agriculture, Omar Al-Mukhtar University, Libya

et al., 2002; Be and Debergh, 2006; Hamad and Taha, 2008c; Akin-Idowu, *et al*., 2014; Nelson, *et al*., 2015) as well as in combination with auxins. Combinations of three hormones, BAP, NAA, IBA (Khatun, *et al*., 1997), KN, NAA and IBA (Mathew and Rangan, 1976; Rahman, *et al*., 2001; Soneji, *et al*., 2002), KN, NAA, IAA (Mathew and Rangan, 1981), BAP, IAA and IBA (Teixeira, *et al*., 2006), two hormones BAP and IAA (Gangopadhyay, *et al*., 2005; Hamad and Taha, 2008 a and b; Dutta *et al*., 2013;) , BAP and NAA (Kofi and Adachi, 1993; Escalona *et al*. 1999; Vesco, *et al*., 2001; Firoozabady and Gutterson, 2003; Perez, *et al*., 2012; Scherer *et al*., 2013), and KN and NAA (Fitchet, 1990) were used for *in vitro* multiplication of pineapple.

Comparison of KN and ZN (Hamad and Taha, 2003), and BAP and KN (Omokoio, *et al*., 2001) and BAP and NAA (Zuriada, *et al*., 2011; Usman, *et al*., 2013) were made but at narrow concentration range. The effect of singly applied IAA, on the other hand, were neither reported at one fixed nor at different concentrations. Using of stationary liquid culture and application of single hormone is cheaper and simpler than solid medium with combined application of two and three hormones. In addition, it is well known that singly applied hormone could, depending on the applied concentrations, promote or suppress the *in vitro* shoot formation. Hence, when few concentrations are used for investigation of hormone effect, it is very likely that the best concentration may left out of comparison. The objective of this study is to compare the effect of two cytokinins (BAP, KN) and two auxins (NAA, IAA) applied singly each at 10 different concentrations on the *in vitro* shoot formation and elongation of Moris pineapple on stationary liquid MS medium for two months of incubation.

MATERIALS AND METHODS

Full strength MS medium (Murashige and Skoog, 1962) were prepared, enriched with sucrose at 20 g/l and divided into 41 glass jars (15 x 5 cm.) each received 30 ml of the medium. The jars were divided into five groups. The first group consisted of one jar, the second, third, fourth and fifth group each consisted of 10 jars. No hormone were added to the first group of jars and BAP at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/l were added to the second group, KN, NAA and IAA each at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0 mg/l were added to the third, fourth and the fifth group of jars respectively. The medium of each jar was adjusted to pH 5.0. The jars were closed with plastic lid and the media were autoclaved at 121 $^{\circ}$C and 1.5 kg/cm^2 for 25 minutes. The content of each glass jar (30 ml) was dispensed under laminar cabinet into 3 sterilized culture tubes (10 ml per culture) using sterilized syringe. Shoots obtained from Moris stock cultures were cultured at density of two shoots per culture tube. After two months of incubation under constant temperature of 25 $^{\circ}$C and 16 hours of light provided by cool white fluorescent lamp, the multiple shoots complex was picked out of the culture tubes, placed over squared paper and separated into individual shoots for counting the shoots and measuring their length. The total shoots per culture were divided by two (explants density) to get the shoot formation per explant and the sum of the shoots length divided by the total shoots to get the average length of shoot. The data were subjected to two ways analysis of variance (hormones and concentration) and the significance of the mean of the different treatments were screened by Duncan Multiple Range Test at $p \leq 0.05$ using SPSS statistical package No. 11.

RESULTS

Analysis of variance showed significant differences between hormone types ($p \leq 0.000$), concentrations ($p \leq 0.003$) and significant interaction between the two factors ($p \leq 0.005$). Average overall concentrations showed that BAP and NAA resulted in highest shoot formation (5.7 and 5.9 shoots) and KN in intermediate shoot formation (4.6 shoots) and each of these hormones resulted in shorter shoots (16 mm).

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Explants treated with IAA produced the fewest (3.8 shoots per explant) but longest (34 mm) shoots (Table, 1). Of all combinations, the highest shoot formation (9 shoots) was obtained in medium enriched with NAA at 4.5 mg/l and the lowest (2 shoots) was in medium enriched with KN at 4.0 mg/l while the tallest shoots (56 mm) was obtained in medium enriched with IAA at 2.5 mg/l and the shortest shoots (6 mm) in medium enriched with KN at 4.0 mg/l. The shoot formation of the BAP treated explants increased as the BAP concentration increased up to 2.0 mg/l and declined afterwards. None of the BAP concentrations resulted in shoot formation less than that of hormone free medium and only one concentration was as effective as hormone free medium (Table, 1).

BAP concentrations could be divided into five different groups. The first and second groups included only one concentration each (2.0 and 3.5 mg/l) and resulted in higher shoot formation (8 and 7 shoots respectively) than the other BAP concentrations. The third group consisted of 3 (BAP at 1.0, 3.0 and 5 mg/l) and the fourth group consisted of 4 (0.5, 2.5, 4.0 and 4.5 mg/l) concentrations and each group resulted in formation of 5 and 6 shoots per explant respectively. The fifth group consisted of one concentration (1.5 mg/l) and the shoot formation was not different from that of hormone free medium (4 shoots per explant). The shoot formation of KN treated explants increased as the KN concentrations increased up to 3.0 mg/l and decreased afterwards. KN concentrations could be divided into three groups. Five concentrations (0.5, 1.0, 2.0, 2.5, and 3.5 mg/l) resulted in more (5 to 7 shoots) shoots than hormone free medium. Three concentrations of KN (1.5, 3.0 and 5.0 mg/l) was as effective as hormone free medium (4 shoots per explant) and two concentrations (4.0 and 4.5 mg/l) produced less shoots (2 and 3 shoots) than hormone free medium. Compared to other KN concentrations, KN at 2.5 mg/l was the best resulting in highest shoot formation (7 shoots) among the KN treated explants.

The shoot formation of NAA treated explants increased as the NAA concentrations increased up to 4.5 mg/l. In fact, the highest shoot formation of all combinations of hormone and concentration (9 shoots) was obtained in medium enriched with NAA at 4.5 mg/l. The NAA concentrations could be divided into 6 groups. NAA at 3.5 and at 4.5 mg/l was the best resulting in 8 and 9 shoots respectively. NAA at 4.0 and 5.0 mg/l resulted in 7 and NAA at 2.0 mg/l resulted in 6 shoots while NAA at 0.5, 1.5 and 2.5 mg/l resulted in formation of 5 shoots. NAA at 1.0 and 3.0 mg/l each resulted in formation of 4 shoots and was not different from hormone free medium. None of the NAA concentrations resulted in shoot rate less than hormone free medium and only 2 concentrations were as effective as hormone free while 8 resulted in more shoots than hormone free medium. The highest shoot formation of the IAA treated explants not only did not exceed 5 shoots, but also required application of highest IAA concentrations (4.5 and 5.0 mg/l). The other concentrations (0.5, 2.5, 3.0, 3.5 and 4.0 mg/l) and (1.0, 1.5 and 2.0 mg/l) resulted respectively in formation of less and equal shoots as that of hormone free medium (3 and 4 shoots). On the other hand, the tallest shoot (56 mm) of all combinations was obtained in medium enriched with IAA at 2.5 mg/l and the shortest (6 mm) was obtained in medium enriched with KN at 4.0 mg/l (Table, 1). All concentrations of BAP and all of the NAA except at 3.0 mg/l and KN except 1.0 mg/l resulted in shoot shorter (two time less) than hormone free medium. On the contrary, IAA at 4 concentrations (low and high) had no effect and at the other 6 concentrations (intermediate) promoted the shoot elongation. The shoot length increased as the IAA concentration increased to a peak of 56 mm long at 2.5 mg/l (two times the shoot length in hormone free medium) and then declined afterward.

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Table (1). Effect of hormone types and concentrations on the *in vitro* proliferation and growth of Moris pineapple in liquid full strength MS medium

Conc. (mg/l)	Hormones types				
	BAP	KN	NAA	IAA	Average
Shoots per explants					
0	4 cde	4 cde	4 cde	4 cde	4 C
0.5	6 abcd	5 bcde	5 bcde	3 de	4.75 ABC
1	5 bcde	6 abcd	4 cde	4 cde	4.75 ABC
1.5	4 cde	4 cde	5 bcde	4 cde	4.25 C
2	8 ab	5 bcde	6 abcd	4 cde	5.75 AB
2.5	6 abcd	7 abc	5 bcde	3 de	5.25 ABC
3	5 bcde	4 cde	4 cde	3 de	4 C
3.5	7 abc	6 abcd	8 ab	3 de	6A
4	6 abcd	2 e	7 abc	3 de	4.5 BC
4.5	6 abcd	3 de	9a	5 bcde	5.75 AB
5	5 bcde	4 cde	7 abc	5 bcde	5.25 ABC
Average	5.7 A	4.6 B	5.9 A	3.8 C	
Shoot length (mm)					
0	27 cdefgh	27 cdefgh	27 cdefgh	27 cdefgh	27 A
0.5	13 ghi	17 fghi	14 fghi	22 cdefghi	16.5 CD
1	11 ghi	32 bcdef	20 defghi	25 cdefgh	22 ABCD
1.5	17 fghi	23 cdefghi	17 fghi	35 bcde	23 ABC
2	11 ghi	17 fghi	12 ghi	29 cdefg	17.2 BCD
2.5	14 fghi	10 hi	14 fghi	56 a	23.5 ABC
3	14 fghi	18 efghi	23 cdefghi	46 ab	25.25 AB
3.5	9 hi	12 ghi	11 ghi	29 cdefg	15.25 CD
4	12 ghi	6 i	12 ghi	25 cdefgh	13.75 D
4.5	11 ghi	9 hi	12 ghi	36 bcd	17 BCD
5	18 efghi	11 ghi	14 fghi	39 bc	20.5 ABCD
Average	14.3 B	16.5 B	15.9 B	33.5 A	

Data were means of 6 explants cultured at density of two explants on culture tubes containing 10 ml of liquid full strength MS medium supplemented with sucrose at 20 g/l and incubated for 60 days under constant temperature and 16 hours of light provided by cool white fluorescent lamps. Mean followed by same letters were not significantly different according to Duncan Multiple Range Test at $p \leq 0.05$.

DISCUSSION

Indole acetic acid (IAA) is definitely out of choice for *in vitro* shoot formation but was the best of the tested hormones for shoot elongation of Moris pineapple in stationary liquid full strength MS medium (Table, 1). None of IAA concentrations resulted in more shoots than in any of the other three tested hormones (BAP, KN, NAA). In addition, out of ten concentrations only two of IAA concentrations resulted in more (promoted) while three in equal (no effect) and 5 in less shoot formation (inhibited) than hormone free medium. The possibility that IAA may promote *in vitro* shoot formation not only was very low (20 %) but also high percentage (50%) of the concentrations inhibited the process of shoot formation. Any of the other hormones (BAP, NAA and KN) could be claimed better than the other for *in vitro s*hoot formation of Moris pineapple depending on how wide the range of tested concentrations was and which concentrations included in that range.

Comparing the three hormones each at equal concentrations (Table, 1) showed that if the tested concentrations were limited to two (1.0 and 2.5 mg/l), KN would be the best hormone while if limited to three (0.5, 2.0 and 3.0 mg/l), BAP would be the best hormone and if limited to five (1.5, 3.5, 4.0, 4.5 and 5.0 mg/l), NAA would be

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the best. Limiting the comparison to each two of the three hormones showed that BAP was better than KN at seven, equal at one and less at two, better than NAA at five and less at five concentrations. Similar, NAA better than BAP at five and less at five, better than KN at six, equal at two and less at two. KN, on the other hand, was better than BAP at two, equal at one and less at seven and better than NAA at two, equal at two and less at six concentrations. (Usman, *et al*. 2013; Zuraida, *et al*. 2011) reported that either BAP or NAA could be better than the other for *in vitro* multiplication of pineapple depending on which concentrations was compared to. (Omokoio *et al.* 2001) found that KN at 4 mg/l was better than BAP while (Fitchet, 1990) reported that KN at 2 mg/l was better than BAP and ZN for Smooth cayenne. On the contrary, Table (1) showed that KN at 4 mg/l resulted in the lowest rate (2 shoots) and at 2.0 mg/l BAP was better than KN. (Aydieh *et al.* 2000; Almeida *et al.* 2002; Fernando, 1986) tested BAP for Queen, Perola and Mauritius pineapple and recommended different concentrations of BAP, 2.0, 1.5 and 1.0 mg/l respectively. This study showed that BAP at 2.0 mg/l was the best for Moris while at 1.5 mg/l had no effect and at 1.0 mg/l induced little effect on shoot formation. The highest shoot formation (9 shoots) obtained using NAA at 4.5 mg/l (Table,1). BAP at 2.0 mg/l was the second best treatment with 8 shoots per explant and would be favored over using NAA at high concentrations (4.5 mg/l). IAA at 2.5 mg/l resulted in the tallest shoots (56 mm). (Firoozabady and Gutterson, 2003) suggested two-stage system for *in vitro* multiplication of Smooth cayenne pineapple. One for shoot formation using BAP at 3.0 mg/l and the other for shoot elongation using combination of BAP at 1.0 and GA_3 at 1.0 mg/l. According to this study two-stage system would also be recommended for Moris pineapple but using different hormones and concentrations. BAP at 2.0 mg/l would be recommended for shoot formation and IAA at 2.5 mg/l for shoot elongation. The contradicting reports about which hormone type or concentration was the best may be due to the incorrect selection of the concentrations for comparison of different hormones. It is well known that hormone could induce three different kinds of effect on organ growth (promote, inhibit and no effect) depending on hormone concentration and type of organ. Nevertheless, the comparison of different hormones was usually based on equal concentrations rather than the kind of effect that concentrations could induce on the growth. Out of ten, nine of BAP and eight of NAA concentrations resulted in more shoots than hormone free (promoted).

That is 90 % and 80 % of the used concentrations of BAP and NAA promoted shoot formation process. For KN, 5 concentrations resulted in more (promoted), three in equal (no effect) and two in less (inhibited) shoot formation than hormone free. That means the chance of KN for promoting shoot formation is only 50 % while 30 % had no effect and 20 % of KN inhibited the shoot formation process. We called these concentrations which resulted in more, equal and less shoots than hormone free medium "promoting", "no effect" and "inhibiting" concentrations respectively and divided each concentration range of similar effect into most and least promoting, and most and least inhibiting. Most of the concentrations that make one hormone better than the other are related to whether the so called most promoting or inhibiting range of that hormone were used. If the concentrations were within the most promoting concentrations of one hormone but within the least promoting or inhibiting concentrations of the other, the comparison from the beginning is in favor of one over the other. For instance, at 5 concentrations (0.5, 1.0, 2.0, 2.5 and 3.0 mg/l) BAP resulted in more shoots than NAA while at the other 5 concentrations (3.5, 4.0, 4.5 and 5.0 mg/l) NAA resulted in more shoots than BAP. That means the possibility of which one of these two hormones is better than the other is 50%. Similar, if the comparison of KN and IAA were limited to two concentrations (4.0 and 5.0 mg/l), IAA would be better than KN. In other words, for valid assessment of hormone suitability for *in vitro* shoot formation, the promoting and inhibiting concentration range of each hormone should be first determined and the comparison limited to the

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most promoting concentration range of each hormone.

The results (Table, 1) showed also that there are some cases in which different hormones could result in equal shoot formation at different and at equal concentrations, and different concentrations of the same hormone could result in equal rate. This indicated that the process of shoot formation could be run using different alternatives. That is the increase in concentration of one hormone and the decrease in concentration of other could compensate for lower or higher shoot formation obtained when equal concentration of the different hormones were used.

Obtaining of equal rate of shoot formation at equal concentration of different hormones did not mean that the different hormones have equal effect. Instead, one of the medium components have reacted differently with different hormones in such way that lowered the effect of one hormone and increased the effect of the other to the point that the two hormones resulted in equal shoot formation. According to this study we suggested that the range of concentration used for testing hormone effectiveness for *in vitro* multiplication should be more than six concentrations. If fewer concentrations were used, it is very likely that the best concentration might be left out of comparison. In addition, for comparison of hormones, the range of promoting, inhibiting and those with no effect should first be determined and then the comparison is limited to concentration ranges of similar effect. However, elucidation of the hormone role could not be made unless concentrations which induced equal and those which induced contrading effect were first identified. Selection of equal concentrations of different hormones that induced promoting effect of one hormone but inhibiting effect of other and different concentrations of same hormone that induced equal effect and testing of their effect in medium of different pH adjustments, medium strength, volume and type accompanied with histological and biochemical analysis would be usefull for elucidation the mechanism of hormone effect.

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تأثير نوع وتركيز الهرمون علي تضاعف ونمو عزلة أناناس (*Ananas comosus* (L.) Merr.) صنف موريس في بيئة موراشيج وسكوق السائلة

عبد الحميد مختار حمد

قسم البستنة، كلية الزراعة، جامعة عمر المختار البيضاء- ليبيا

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المستخلص : اختبر تأثير عشرة تركيزات (0.5 ، 1.0 ، 1.5 ، 2.0 ، 2.5 ، 3.0 ، 3.5 ، 4.0 ، 4.5 ، 5.0) لنوعين من السيتوكينين (BAP و KN) ونوعين من الأوكسين (NAA و IAA) على نموات عزلة لنبات الأناناس صنف موريس في وسط موريشيج وسكوق (MS) السائل المحتوي على 20 جرام في اللتر وحموضته (pH) 5.0 ، استعمال هرمون (Naphthalene Acetic Acid (NAA أعطى أعلى عدد نموات للعزلة الواحدة (9 نموات) ، العدد الأعلى الثاني (8 نموات) تم الحصول عليها في وسط يحتوي على (BAP) بتركيز 2.0 مليجرام في اللتر ، من بين عشرة تركيزات اختبرت في هذه الدراسة فإن (NAA) كان أفضل من (BAP) في التركيزات الخمسة الأعلى وأقل في حالة التركيزات الخمسة الأدنى لكن في كل التركيزات العشرة كلا الهرمونين أدى إلى نموات أكثر مما لو استعمل في وسط خالٍ من الهرمون ، ما عدا تركيزين فأن هرمون BAP و NAA كان أفضل من هرمون KN، لهذا يمكن القول أن أي من هذه الهرمونات الثلاثة KN و NAA و BAPمن الممكن أن يعتبر أفضل من الهرمونين الاخرين اعتماداً على التركيزات التي تستعمل في المقارنة بينها، من الناحية الأخرى فإن هرمون IAA لا يصلح لزراعة عزلات الأناناس إذا كان الوسط في حالة سائلة، حيث إن كل التركيزات العشرة من هرمون IAA أعطت نموات أقل مما تم الحصول عليه من استعمال الوسط الخالي من الهرمون ، لكن استعمال هرمون IAA بتركيز 2.5 مليجرام أدى للحصول على أطول نمو (56 مليمتر) وسبعة تركيزات أدت إلى نموات أطول من تلك في الوسط الخالي من الهرمون (27 مليمتر) في حين كانت أطوال النموات في الأوساط المحتوية على أي من الهرمونات الثلاثة الأخرى أقل من نصف الطول في الوسط الخالي من الهرمون، بهذا فأن هرمون BAP بتركيز 2.0 مليجرام كان أفضل معاملة لمضاعفة عدد النموات من العزلة الواحدة وبينما هرمون IAA بتركيز 2.5 مليجرام كان أفضل معاملة للحصول على أطول نمو .

الكلمات المفتاحية: الأناناس، المزرعة السائلة، مضاعفة نموات العزلة.

عبد الحميد مختار حمد : abdelhamidhamad@gmail.com ، كلية الزراعة، جامعة عمر المختار البيضاء- ليبيا.