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Effect of 6-Benzylaminopurine and α-Naphthalene Acetic Acid on *In vitro* Production of MD2 Pineapple Planting Materials

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Abstract: MD2 pineapple is currently the most preferred variety on the international market pleasant aroma and high brix acidity ratio. Large quantities of planting material are needed to replace the existing Smooth Cayene and Sugar Loaf varieties commonly grown by farmers. Therefore in vitro procedures were developed as an alternate method to improve upon the multiplication rate of this variety. Clumped explants were cultured on MS supplemented with 30 g/l sucrose and various combination of 6-benzylaminopurine (BAP) and α-naphthalene acetic acid (NAA) in solid or liquid cultures. Liquid cultures required 5.0 mg l^{-1} BAP to significantly ($P<0.05$) increase the multiplication rate by 2- or 5-fold compared to 7.5 mg l^{-1} in solid cultures. When plantlets from optimal treatments were transferred to liquid medium with high concentration (7.5-15 mg l^{-1}) of NAA there was no root development. However, the transfer of one or two-month old cultured plantlets to solid MS medium supplemented with NAA or IBA alone or combination of NAA and IBA resulted in root production. The number of root produced per plantlet on a medium supplemented with a combination of NAA and IBA was comparably higher than when NAA or IBA alone were used. The increased multiplication rate of MD2 in vitro will serve as alternate source of planting materials for both subsistence and large-scale farmers in the pineapple industry.

Key words: Pineapple · *in vitro* · multiplication rate · 6-Benzylaminopurine · α-naphthalene acetic acid

INTRODUCTION

The introduction of MD2 variety into the fresh pineapple market in 1990s has increased its demand in both the USA and Europe. This pineapple variety bred in Hawaii by Pineapple Research Institute in 1970 [1] is currently the most preferred on the international market compared to the Smooth Cayene; it controls about 75% of the European Union market [2]. The MD2 variety has uniform fruit size with solid golden yellow pulp and a very pleasant aroma when ripe with longer shelf life than Smooth Cayenne. Furthermore, it has high Brix (14 and more) with low acidity 0.4-0.45%, thus it has a good Brix acidity ratio of 25 or more [3]. Consequently, there is a gradual shift from export of Smooth Cayenne to MD2 by most pineapple farmers.

However, the large numbers of propagules required for cultivation of the MD2 variety (60,000 per hectare) in order to meet the international market demand cannot be obtained via traditional propagation of slips, suckers, crowns, or hapas. Besides, the importation of suckers for direct planting by farmers is also expensive and may not meet quarantine requirements. Thus, *in vitro* multiplication would be a useful alternative for mass production of MD2 to commercial farmers.

In vitro propagation of pineapples for plantlet regeneration [4,5] and conservation [6] is well documented. It has comparative advantage over the traditional methods as it leads to the production of large numbers of disease-free uniform planting materials in a relatively shorter period independent of the season. DeWald *et al*. [7] and [4] have independently reported of increased multiplication rate of Smooth Cayenne by 40- to 85- fold over a period of 13 months by culturing crown meristem explants on a medium supplemented with BAP: further increase has been achieved by the use of the temporary immersion system [8] and bioreactors [5].

However, *in vitro* multiplication of the MD2 variety should be cost effective and affordable so as to be able to

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compete with field-grown suckers. Cost reduction could be achieved by increasing the multiplication rate through manipulation of BAP and NAA concentration in the culture medium. According to [9], it is possible to produce 6,575 pineapple on a medium supplemented with benzylaminopurine (BAP). In this paper, we report on the effect of BAP and NAA on *in vitro* multiplication of MD2 pineapple. The effect of liquid or solid culture medium on the multiplication rate as well as the effect of NAA and/or IBA on rooting is also reported.

MATERIALS AND METHODS

Plant materials: *In vitro* plantlets of MD2 pineapple variety imported from Costa Rica were obtained by the kind courtesy of Dr. H.M. Amoatey. These were multiplied on [10] basal salt and vitamins (MS) medium supplemented with 30 g/l sucrose, 100 mg l^{-1} myoinositol and 4.5 mg l^{-1} BAP and 0.7 mg l^{-1} NAA on 6 to 8 weeks subculture regime. All media were adjusted to pH 5.7 prior to autoclaving. Cultures were incubated at a temperature of 26°C and light intensity of 2800 lux. Eight-week old plantlets were used for all the experiments.

BAP and NAA on multiplication rate of *in vitro* explants: Eight-week old basal portions of pineapples consisting of two or three proliferating buds (clumped explant) were cultured in liquid or solid MS supplemented with 30 g/l sucrose, 100 mg l^{-1} myoinositol varying concentrations of BAP (2.5-7.5 mg l^{-1}) and NAA (1-4 mg l^{-1}) (Table 1). Twenty (20 ml) of solid medium in Magenta vessels was used while for liquid media only 10 ml in Erlenmeyer flask was used to avoid total immersion and subsequent suffocation of the explants. Ten explants were used per treatment were labelled as A, B or C (Table 1). Liquid cultures were placed on an orbital shaker while solid cultures were placed on shelves in the growth room. Incubation conditions were as described above. After six weeks of culture, the number of plantlets generated per clumped explant in both liquid and solid cultures was recorded.

Higher concentration of NAA on root induction in liquid medium: Clumped explants obtained from optimal treatment (C_2) treatment above were cultured in a liquid medium supplemented with 7.5 mg l^{-1} BAP and increased concentrations of NAA (7.5-15.0 mg l^{-1}) to induce root formation while cultures were in the liquid multiplication medium. The cultures were again placed on an orbital shaker and incubated under growth room conditions as previously described. The number of plantlets regenerated with roots was counted six weeks after culture.

NAA and/or IBA on root induction on solid medium: One or two month-old cultured plantlets were carefully trimmed at the base and cultured on 25 ml of solid MS supplemented with 30 g/l sucrose, 100 mg l^{-1} myoinositol and either 0.5 mg l^{-1} NAA or 0.5 mg l^{-1} IBA or a combination of 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} IBA or 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} IBA (Table 2). Cultures were incubated in the growth room as described above. The number of explants with roots was recorded 6 weeks after culture. After additional 2 weeks, rooted explants were washed off the agar and transferred to jiffy pots for hardening. Well established plantlets were transferred to the greenhouse for growth and subsequent field establishment.

Statistical Analysis: All statistical analysis was performed using Micosoft Excel Statistical Tool Pack.

RESULTS

Effect of BAP and NAA on *in vitro* proliferation rate: All BAP with NAA treatment combinations in both liquid and solid medium resulted in the proliferation of shoots without roots from cultured explants with the exception of treatment A_2 and A_3 (Table 1). Also, with the exception of treatment A_2 and independent of solid or liquid medium the number of plantlets produced was optimal when NAA concentration in the medium was 2.0 mg l^{-1} and the BAP concentration was two and half times higher than NAA. For liquid medium, the best BAP with NAA treatment combination was B_2 where there was significantly (P=0.05) higher shoot production (29.3 shoots per clump) than the remaining treatments except in treatment C_2. In contrast, the best treatment for solid medium was C_2. The optimal treatment for both solid and liquid cultures was 7.5 mg l^{-1} BAP with 2.0 mg l^{-1} NAA. In this treatment combination 28.5 and 16.1 shoots were produced per clumped explant in liquid and solid medium respectively.

Effect of higher NAA concentration root production in liquid culture: After the determination of the optimal BAP concentration (7.5 mg l^{-1}), the NAA concentration was increased from 7.5mg l^{-1} to 15 mg l^{-1} with the aim of inducing roots while cultures were still proliferating in liquid medium. The results are as shown in Table 2. There was rapid shoot proliferation in all the cultures

Table 1: Effect of varying concentration of BAP and NAA on multiplication of pineapple plantlets in liquid and solid medium

	Concentration of		Mean no. of plantlets produced on	
Treatment	BAP (mg l^{-1})	NAA (mg l^{-1})	Liquid	Solid
A_1	2.5	1	11.2±2.5a	9.5±1.0a
A_2	2.5	2	0.0±0.0c	0.0±0.0c
A_3	2.5	4	0.0±0.0c	0.0±0.0c
B_1	5.0	1	10.0±0.5a	8.7±1.6a
B_2	5.0	2	29.3±3.1b	7.4±1.6a
B_3	5.0	4	13.0±1.4a	9.7±1.1a
C_1	7.5	1	7.2±0.7a	14.1±3.5a
C_2	7.5	2	28.5±4.3b	16.1±2.6ab
C_3	7.5	4	10.4±1.4a	9.9±1.2a

Treatment means in a column followed by different letters are significantly different at P< 0.05 according to Tukey's test

Table 2: Effect of higher NAA concentration on shoot proliferation of MD 2 pineapple clump explant in liquid culture. Explants were obtained from optimal treatment above

BAP/mg l^{-1}	NAA /mg l^{-1}	Mean number shoots
7.5	7.5	42.8a
7.5	10.0	12.3b
7.5	15.0	7.3b

Means in a column followed by different letters are significantly different at P< 0.05 according to Tukey's test

Fig. 1: Effect of 7.5 mg l^{-1} BAP and higher concentrations of NAA on shoot proliferation in a liquid culture medium

independent of the NAA concentration but there was no root development (Fig. 1). However, the number of shoots produced significantly (P<0.05) reduced, as the concentration of NAA in the medium increased indicating that high concentration of NAA is detrimental to shoot proliferation. Explants cultured on a medium with 15 mg l^{-1} NAA resulted in the production of compact clumps with plantlets having shorter leaves. Increasing the NAA concentration by one and half times to 10 mg l^{-1} resulted in more than three fold reduction in number of shoots produced.

Effect of NAA and/or IBA on root induction on solid medium: The results of the effect of NAA and/or IBA on root induction on solid medium are shown in Figs. 2 and 3. All cultured shoots developed roots one week after culture independent of the age as well as the growth regulator treatment. Root development continued until the sixth week when cultures were transferred to the soil. Figure 3 shows a comparison of the mean number of roots produced per plantlet for one-month and two- month old cultures at the end of the sixth week of culture. In general, rooting medium supplemented with a combination of NAA and IBA induced more roots than NAA or IBA alone supplemented medium. Also, the number of roots developed increased as the concentration of NAA and IBA in the rooting medium doubled. Analysis of variance (ANOVA) showed significant (P=0.046) and highly significant (P=0.0001) differences in the mean number of roots produced per plantlets for one-month-old and two-month-old cultures respectively. The optimal medium for rooting was MS supplemented with 1.0 mg l^{-1} NAA and 1.0 mg l^{-1} IBA where for two-month old cultures the mean number of roots (15.6) developed after the sixth week was almost twice that of 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} IBA. All rooted plantlets survived hardening in Watson modules filled with topsoil and subsequent transfer to black polyethylene bags for growth (Fig. 4).

DISCUSSION

The high planting density of 60,000 pineapples per hectare requires an efficient and rapid planting material production system to meet farmers demand. Thus, the application of *in vitro* technique which ensures rapid disease-free planting materials is an appropriate alternative which has been documented by many authors [9,11]. It has been estimated that 6,575 *in vitro* pineapple plantlets could be produced on an MS medium supplemented with 3 mg l^{-1} BAP [9]. In this report, the optimal treatment for MD2 pineapple plantlet production in both liquid and solid medium was an MS medium

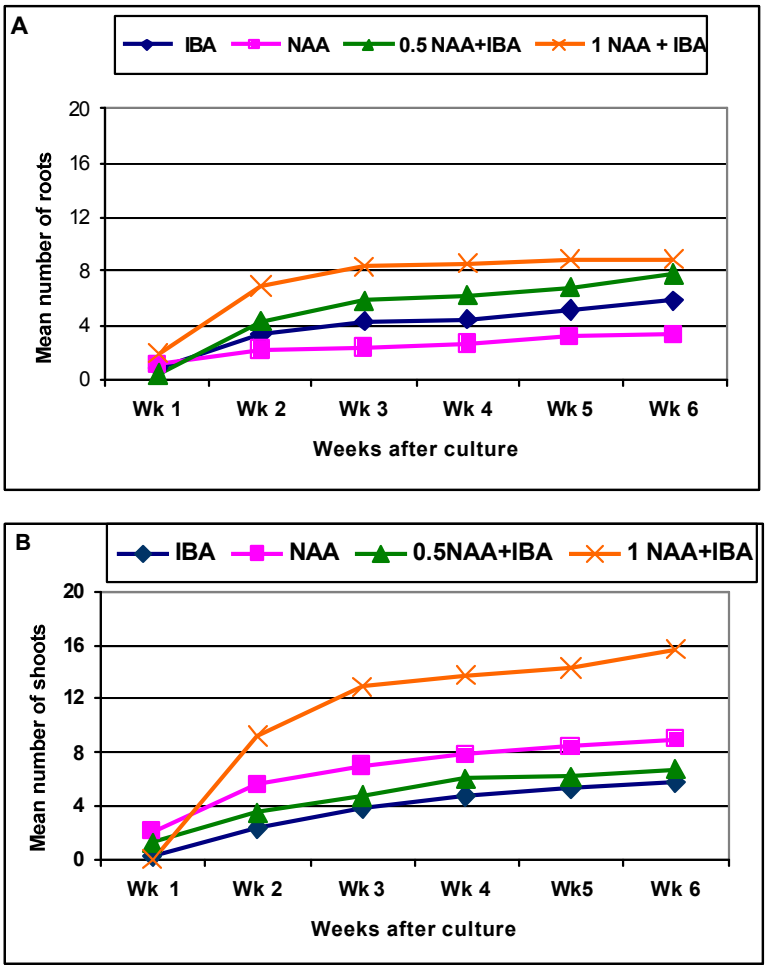

Fig. 2: Effect of NAA and IBA on root induction from one month (A) and two months (B) old cultures

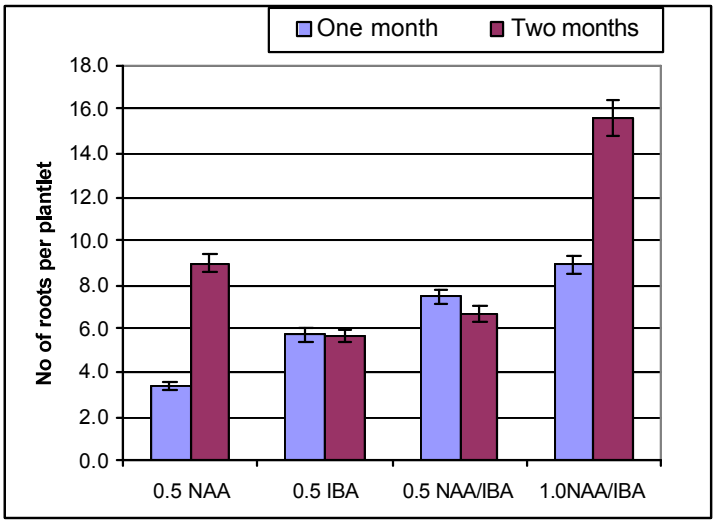

Fig. 3: Mean number of shoots produced by plantlets at the end of six weeks of culture

Fig. 4: Rooted MD2 pineapple plantlets growing in pots filled with top soil in the greenhouse

supplemented with 7.5 mg l^{-1} BAP and 2 g/l NAA which resulted in the production of 28.5 and 16.1 pineapple plantlets respectively in only one subculture. However, in liquid medium lower concentration of BAP (5.0 mg l^{-1}) lead to a significantly (P=0.05) higher production of MD2 pineapple plantlets (29.3 plantlets) than solid cultures (7.5 mg l^{-1}). The results presented here confirm the assertion that generally, liquid culture results in faster rate of growth than solid cultures [12]. The faster rate of growth in liquid cultures may be attributed to exposure of greater surface of the explant to the medium therefore enhancing nutrient uptake due to its uniform distribution [13]. It has also been shown that liquid medium disperses phenolic exudates from the explants consequently resulting in faster growth [14]. In this study, the optimal concentration of 5.0 mg l^{-1} BAP in liquid cultures increased shoot production by almost four-fold indicating faster growth and production than solid cultures. Firoozabady and Gutterson [5] have similarly reported of higher multiplication rate of pineapple cv Smooth Cayene in liquid cultures than solid cultures. They estimated that further increases of 3000 fold in multiplication rate could be achieved by using periodic immersion bioreactor (PIB).

A cost benefit *in vitro* production of MD2 pineapple will be simultaneous shoot proliferation with root development, which will reduce steps in production as well as labour cost. Thus, we increased the NAA concentration from 7.5 mg l^{-1} to 15.0 mg l^{-1} while maintaining a BAP concentration of 7.5 mg l^{-1} in order to induce rooting while shoots were proliferating liquid cultures. In spite of this treatment, there was no root formation but rather there was rapid shoot multiplication resulting in clump formation. The number of shoots per clump, however, declined as the concentration of NAA in the medium increased. Contrary to our results, [5] obtained roots from liquid cultures of pineapple cultured on MS medium supplemented with 0.5 mg l^{-1} NAA and 0.5 mg l^{-1} IBA. The differences in these two results might be due to the presence of cytokinin BAP in our culture medium and the absence of IBA. Cytokinins are known to stimulate cell division and axillary bud proliferation [14] thereby resulting in shoot formation at the expense of root development.

Root development was rather achieved on solid MS medium supplemented with NAA, IBA or combination of NAA and IBA. The NAA and IBA combination treatment produced comparatively higher roots than either NAA or IBA alone and was also depended on the age of plantlets before rooting. NAA and IBA are root inducing growth regulators and have been used either alone or in combination for root initiation in many cultures [12]. The marked improvement in mean number of roots produced when NAA and IBA were applied in combination may have resulted from these growth regulators acting either in concert or synergistically to induce roots. The increased root production is beneficial, as it will enhance post flask acclimation survival and subsequent field establishment of the plantlets. Almost all rooted plantlets survived acclimatisation in jiffy peat pots and subsequent growth in polyethylene bags (Fig. 4). This study have shown that large-scale commercial production of MD2 pineapple planting materials *in vitro* could be achieved by using lower concentration of BAP in liquid cultures. The improvement in the production of planting materials may result in the reduction of cost of production of MD2 planting materials which augurs well for both commercial and subsistence farmers who need this variety to replace the existing Smooth cayenne variety, which is phasing out on the international market.

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