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Meta-topolin for Pineapple Shoot Multiplication under Three *In vitro* Systems

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Abstract: Meta-topolin is a new cytokinin for micropropagation. The present investigation assessed response of pineapple shoot proliferation for meta-topolin (mT) using solid media, shaking liquid media and temporary immersion system (TIS). In each culture system, 2.5 or 5 μM mT with or without 2.5 μM NAA were compared with the MS basal medium. After six weeks, the medium with 2.5 μM mT led to the highest shoot proliferation in shaking liquid system. While the one with 5 μM mT was the best for both solid and TIS. The hourly immersion time for 4 and 6 minutes in the TIS gave the non-significant result. When the best media with mT of the three culture systems were compared for 6 weeks, the shaking liquid medium with 2.5 μM mT yielded the best growth and the highest multiplication of 11.96 folds.

Key words: Aromatic cytokinin · Antagonist · Culture medium · Semi-solid · Liquid · Temporary immersion system

INTRODUCTION

Cytokinins play important roles in cell division, differentiation and allocation of nutrients, inhibition of senescence, activation of axillary meristems and inhibition of cell elongation [1]. Chemically, natural cytokinins are N^6-substituted purine derivatives which can be classified by the configuration of their N^6-side chain as isoprenoid or aromatic cytokinins. Kinetin and benzylaminopurine (BA) are the best-known cytokinins with ring substitutions at the N^6-position. Cytokinins with an isoprenoid side chain were thought to be endogenous compounds; however, later on BA derivatives were identified as natural cytokinins [2-4]. The hydroxylated forms of BA, meta- and orthotopolin, occur naturally, with the accompanying nucleosides, nucleotides and Oglucosides [3, 5, 6]. Tarkowska *et al.* [7] identified methoxy derivatives of ortho- and meta-topolin (MemT, MeoT) and their 9-ribosides (MemTR, MeoTR) in *Arabidopsis thaliana* and *Populus x canadensis* leaves and their name derived from 'Topol' (name given for Poplar in Czech language).

There are reports describing the application of an aromatic cytokinin, particularly, meta-topolin' (N^6-(meta-hydroxybenzyl) adenine (mT) in micropropagation. The activity of mT was interestingly comparable to that of the most active isoprenoid cytokinin, zeatin in the bioassays [3, 6]. In tissue culture, mT was more effective than BA on shoot and root production of *Spathiphyllum floribundum* cv. Petite [8]. It could speed up multiplication and control of hyperhydricity in *Aloe polyphylla* [9]. It could also improve multiplication on plantain [10] and *Pelargonium hortorum* [11]. Moreover, some reports also indicated that plants grew on a medium with mT performed better than that with equimolar concentration of BA [8, 10, 12].

A number of researchers [13-21] have developed tissue culture methods for propagating pineapple. These researchers have used various types of cytokinin like BA, Kinetin, without or with combination of auxin. Furthermore, the new aromatic cytokinin, meta-topolin, have gotten attention in tissue culture of many plant species, but has not any information in micropropagation of pineapple. In the present communication, we assess response of pineapple multiplication for metatopolin (mT) using different *in vitro* systems, solid media, shaking liquid media and temporary immersion systems.

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MATERIALS AND METHODS

Plant Materials: *In vitro* shoots of a smooth cayenne pineapple (*Ananas comosus* 'Pattawia') on solid medium was used as experimental materials. Plantlets with 2-3 cm height were cut longitudinally. The segment was used as an explant. The explants were placed in the liquid media or on solid media with cut surface downward.

Multiplication Rate of Pineapple Cultured Using Meta-topolin in Three *In vitro* Systems: For all media 2.5 or 5 μM meta-topolin (N^6-(meta-hydroxybenzyl) adenine) (Phyto Technology Laboratories™, USA) with and without 2.5μM NAA were tested using a complete randomized design with four replications. For the solid media, the explants were cultured on the media with 6.5 gm L^{-1} agar-agar in a 100 ml culture vessel. Each vessel was contained 20 ml culture media. For shaking liquid culture, the explants were placed in 100 ml culture vessel containing 20 ml of culture media. The culture vessel was placed on 100 rpm orbital shaker.

For the temporary immersion systems (TIS), a non-air flow single flask on a rotary was used. A rough translucent polycarbonate sheet was folded in 100 ml Erlenmeyer flask to hold an explant above the media temporarily upon rotation. In an immersion cycle, explants were hourly immersed in for 4 or 6 minutes. The 2x5 factorials of immersion times and plant growth regulator were employed.

The basal medium contained Murashige and Skoog salt and vitamins [22] with 3% sucrose. The pH was adjusted to 5.8 with 0.1 N sodium hydroxide 1N potassium hydroxide or 0.1 N hydrochloric acid 1N nitric acid before autoclaving for 20 minute at 121°C and 1.1 kg cm^{-2}.

Cultures were incubated at, 27 ± 2°C under 16 hrs photoperiod from cool white fluorescent lamps. After six weeks, explants were recorded for their number of shoot per explants.

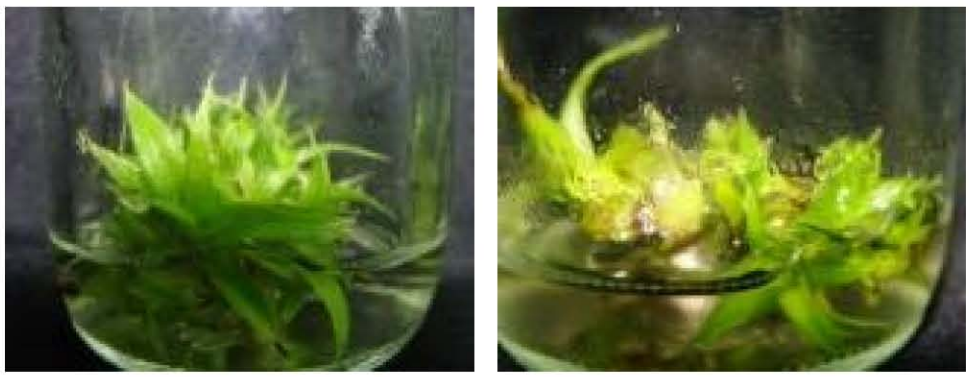
Multiplication Rate of Pineapple Shoot Using Selected Media for the Three *In vitro* Systems: The explants similar to those used in experiment one was transferred to the best media of each *in vitro* system. Each *in vitro* system was replicated 20 times. The number of shoot per explant, height, fresh weight and dry weight after 42 days were statistically compared.

Statistical Analysis: All measurements were subjected to analysis of variance (ANOVA). Means comparisons were preformed using Duncan's multiple ranges test (DMRT). The statistical was done using statistics program [23].

RESULTS AND DISCUSSION

Multiplication Rate of Pineapple Cultured Using Meta-topolin in Three *In vitro* Systems

Shaking Liquid Media: In the shaking liquid media, adding mT led to higher shoot per explants than the basal medium (Table 1). Among the media with only mT, shoot multiplication rate in the medium containing 5 μM mT was statistically the same as that in the control (Table 1). The concentration might be supraoptimal for promoting pineapple growth acceleration. Adding 2.5 μM NAA to the mT, the multiplication rate could be enhanced only in the media with 5 μM mT. However, abnormality growth, specifically swollen and relatively thick leaves were observed (Fig. 1). The shoots indicated probable might be vitrification. It might indicate that the negative effects of



A) Normal B) Abnormal

Fig. 1: Normal and abnormal (hyperhydric) pineapple plantlets from the shaking liquid media with 2.5 μM mT concetration (A) and 5 mT with 2.5 μM NAA (B) after six weeks of culture

Table 1: Multiplication rate of pineapple 'Patawai' cultured in two *in vitro* systems with different metatopolin (mT) and NAA concentration for six weeks

PGR in MS media (µM)			
mT	NAA	Shaking liquid	Solid
0.0	0.0	7.25w	3.25c
2.5	0.0	15.75x	4.00b
5.0	0.0	8.50w	6.75a
2.5	2.5	13.00xy	4.25b
5.0	2.5	11.50y	4.00b
F-test		*	*
C.V. (%)		18.07	18.6

Means followed by the same letter in the same column are not significantly different by Duncan's multiple range test at p=0.05.
PGR= plant growth regulator

Table 2: Multiplication rate of pineapple 'Patawai' using different meta-topolin (mT) and NAA concentrations with two immersion times under temporary immersion systems for six weeks

Interaction effects		
Immersion time	PGR in medium (µM)	Multiplication (fold)
4 minute per hour	0.0 mT + 0.0 NAA	2.5c
	2.5 mT + 0.0 NAA	4.25a
	5 mT + 0.0 NAA	5.0a
	2.5 mT + 2.5 NAA	3.5b
	5.0 mT + 2.5 NAA	3.25b
6 minute per hour	0.0 mT + 0.0 NAA	3.25c
	2.5 mT + 0.0 NAA	3.5b
	5.0 mT + 0.0 NAA	3.75b
	2.5 mT + 2.5 NAA	3.75b
	5.0 mT + 2.5 NAA	2.50c
Plant growth hormone effects		
0. 0mT + 0 NAA	2.87C	
2.5 mT + 0 NAA	3.88AB	
5. 0mT + 0 NAA	4.38 A	
2.5 mT + 2.5 NAA	3.62B	
5. 0mT + 2.5 NAA	2.87C	
Immersion time effects		
4 min per hour	3.7A	
6 min per hour	3.35A	
F-test		
PGR	*	
Time	NS	
Time x PGR	*	
C.V. (%)	16.17	

Means followed by the same letter (small letter for mean of interaction and capital letter for single mean effects) are not significantly different at p=0.05 level of probability using Duncan's multiple range test. NS= Non-significant and * = significant at P=0.05

Table 3: Effects of the three *in vitro* systems on shoot multiplication and shoot growth of pineaple 'Patawai' with sellected meta-topolin containing media

In vitro system	Shoot multiplication	Height (cm)	Diameter (cm)	Fresh weight (gm)	Dry weight (gm)
Liquid media	11.96a	5.00a	0.58a	5.77a	1.78a
Solid medium	8.98c	3.6b	0.58a	2.74b	0.5b
TIS	9.43b	4.85a	0.61a	5.08a	0.5b
F-test	*	*	NS	*	*
C.V. (%)	14.23	8.58	12.11	11.46	18.04

Means followed by the same letter in the same column are not significantly difference by Duncan's multiple range test at p=0.05

the auxin could be due to interaction effects cytokinin [24]. In this experiment the lower mT concentration enhance multiplication of pineapple plantlets. The promotion of mT for shoot multiplication has been demonstrated by [9, 10] on other plant species.

The optimal mT concentration, which produced 15.75 normal shoot per explants with relatively healthy explants was 2.5 µM. Our result was compared with other multiplication of pineapple in liquid media, better than Be and Debergh [15] reported MS media supplemented with 0.8-1 mg l-L of BA resulted 9 shoot cultured interval of 8 weeks and lower than that of Danso *et al.* [14] which obtain 29.3 fold multiplication of MD2 pineapple in 42 days using modified MS liquid medium supplemented with 5 g /l BAP and 2 g/l NAA.

Solid Media: The solid media with mT resulted in significantly higher shoot multiplication rate than the basal medium (Table 1). The multiplication rate was increased with the mT concentration when individually applied. It is thus worthwhile to experiment using higher concentration of mT in the future. Adding 2.5 μM NAA to mT containing media blocked the multiplication enhancing effects of raising mT concentration from 2.5 to 5 μM. The continuous presence of high NAA concentration in the multiplication medium was detrimental to growth and multiplication of pineapple shoot-tip culture [19, 20]. However, Bairu *et. al* [24] reported that optimum and normal shoot multiplication of *Harpagophytum procumbens* was achieved by omitting auxin and using the aromatic cytokinin. Similarly, Palni *et al.* [25] reported that zeatin riboside was degradative metabolites (adenine, adenosine and adenosine nucleotides) formed during the supplement with high concentrations of NAA. This auxin effect on cytokinin metabolism appears to be mediated, at least in part, through cytokinin oxidase. The best concentration for the shoot multiplication in the solid media was at 5 μM mT. It resulted in an average of 6.75 shoots per explants after 42 days.

Temporary Immersion System: Meta-topolin containing media yielded higher shoot multiplication rate than the basal media. When individually applied, both 2.5 μM and 5 μM mT gave statistically the same result (Table 2). The effect of mT on shoot proliferation was less pronounced when 2.5 μM NAA was added. More interestingly, the effect of 5 μM mT on shoot multiplication was nullified by 2.5 μM NAA. The antagonistic interaction between mT and NAA on pineapple micropropagation is thus need future investigation.

The immersion (duration and frequency) for temporary immersion system provides a highly aerobic system for plant growth, which is the most decisive parameter for efficiency of the system [26, 27]. It affects nutrient supply and composition of the internal atmosphere in the culture vessel of air-flow TIS [28]. Jackson [29] stated that an aqueous cover interferes strongly with gas exchange to the outer tissue or cell surface since gas diffusion rates in water are approximately 10,000 times slower than in air. This impact is increased with the depth of the aqueous cover or the inclusion of gel matrices such as agar. Different plant requires different flush and rest time for optimal multiplication [27]. Pineapple multiplication and growth was achieved with immersion time 2 minutes every 3 hours using 200 ml medium in 1 litter of twin bottle for temporary immersion systems [30]. On the other hand, multiplication of pineapple was attained with immersion time at 6 minute in every 25 minute using 10 liters of vessel by periodical immersion bioreactor and indicated that they observed some necrotic effects on the shoots [18]. The present experiment showed that the single effects of hourly immersion time of 4 and 6 minutes were non-significant.

The immersion time, however, showed an interaction with mT concentration on shoot proliferation. The 4 minute immersion time gave better result of multiplication rate than the 6 minute with both mT concentrations. The immersion time intervals play a decisive role in influencing gaseous exchange for photosynthesis and respiration even if there was dissolved oxygen and carbon dioxide in the medium [29]. In our experiment, the hourly immersion time of 4 minutes with the media containing 5 μM mT yielded the highest multiplication rate of 5 folds in 6 weeks. Five folds increase was same as when a smooth cayenne pineapple was cultured on modified MS medium with 1.5 mg L^{-1} BA and 0.5 mg L^{-1} NAA but in 4 weeks [18]. Therefore, the effects of higher concentration of mT with a 4 minute immersion time of might get a future attention.

Multiplication Rate of Pineapple Shoot Using Selected Media for the Three *In vitro* Systems: When the best media for each culture systems were compared for 6 weeks, the shaking liquid media with 2.5 μM mT yielded the best multiplication rate of 11.96 folds followed by the TIS and the solid media with 5 μM mT of 10.43 and 8.98 folds, respectively (Table 3).

The higher shoot production might be probably favored by the better nutrient uptake in the liquid media [13]. The use of liquid media facilitates the nutrient uptake of the shoot due to the distribution in the culture medium. However, hyperhydricity is often present [31]. Bairutal [9] reported that hyperhydricity was minimized by using the 5 μM mT for *Aloe polyphylla* micropropagation.

Our results were contradicted to the finding of Escalona *et al.* [30] which reported that TIS yield the highest pineapple shoot number per explants when compare to liquid and solid media. Their TIS was an air-flow twin bottle system and with 200 ml medium per explants in 300 ml flask. On the other hand, our TIS were a closed system with 30 ml medium per explants in 100 ml flask. The different in the types of TIS media volume as well as the head space could contribute to the multiplication performance. The availability of CO_2 and O_2 as well as the C_2H_4 concentration in the system may play an important role in shoot multiplication [32]. The continuous tissue growth and proliferation was also rapidly limited by the size of the culture vessel [15].

Height of *in vitro* pineapple shoot is very important to minimize loss of proliferation potential during subculture [13]. In this experiment, statistically greater height of shoot was produced in the shaking liquid medium (5 cm) and TIS (4.85 cm) than the solid medium (3.6 cm). However, diameter of shoot was not differently affected by the three systems.

Fresh weight of shoot was lowest on the solid media. However, dry weight in the shaking liquid medium was triple of the other. The highest water content was observed in the shoots from TIS. This apparent contradiction of higher fresh weight and lower dry weight of TIS may also be explained by the reduced development of the leaves in the temporary immersion culture [30]. Generally, solid media was the poorest for multiplication and growth of pineapple. This might be due to the least uptake of nutrient media by the explants. Liquid medium, on the other hand, was the best for multiplication and growth. This might be due to the best contact with the mT media [31].

The rate of the shaking liquid media 11.96 shoots obtained after 6 weeks of incubation (Table 3) is better by 6 fold than that reported by Firoozabady and Gutterson [18] and Ika and Ika [33] and better by 2 fold reported by Be and Debergh [34] but less than that reported by Escalona *et al.* [30] using the TIS. The differences between our results and theirs could be attributed to different in plant growth regulator, subculture frequency and the number of subculture as well as light intensity [35].

CONCLUSION

The present report showed that that pineapple had good response to meta-topolin. It was suggested that the developing plantlets show normal morphological characteristics and better multiplication in the optimum concentration at 2.5 μM mT for shaking liquid media and 5 μM mT for both solid and TIS. In addition, meta-topolin without auxin was observed was better in all the *in vitro* systems but need future investigation. The shaking liquid media were effective in the lower concentration for better shoot multiplication. The liquid media with 2.5 μM mT was better *in vitro* system for effective shoot proliferation than the other.

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