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In-vitro proliferation of *Musa balbisiana* improves with increased vitamin concentration and dark culturing

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ABSTRACT

Musa balbisiana is a wild banana genotype with important traits such as drought tolerance and disease resistance. Uniform and clean plants are often required to study these traits in different laboratories but plants can only be generated through a tissue culture process yet for a long time a protocol for regeneration of the same has not been available. Here, we demonstrate that modification of the anti-oxidant content of the in- vitro plant proliferation medium through adjusting the concentration of ascorbic acid and thiamine HCl in the basal MS medium together with subjecting the explants to dark culturing conditions improved proliferation of *M. balbsiana* by over 10 fold. These treatments resulted in 40 shoots per initial explant material at the best performance.

1. INTRODUCTION

M. balbisina (BB) is a wild banana genotype that has been found to be a potential source of important traits including drought tolerance [1] and disease resistance [2]. Modern techniques for exploring such traits depend on successful isolation of the responsible genes and this often requires to start with clean and uniform plantlets. Such plants are normally generated through a tissue culture process. Initiation and culturing of *M. balbisiana* on a tissue culture medium normally used for multiplication of the East African Highland Banana (EA-AAA) materials [3] often fails as explant materials blacken and die. This was particularly noted during an attempt to generate *M. balbisiana* plantlets for studying its mechanism of resistance to Banana Xanthomonas wilt disease. Studies have shown that the concentration of the synthetic cytokinin, 6-benzyladenine (BA) influences proliferation of a related *M. balbisiana* variety 'Kluai Hin' (BBB genome) [4]. It was also reported that the rate of proliferation is also affected by the degree of browning of the shoot tip tissues which phenomenon is attributed in part to tissue death resulting from oxidative stress incurred at the cut surfaces of tissues or to oxidation of plant phenolic compounds in presence of light [5],[6],[7],[8],[9],[10].

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Death of surface tissues interferes with the tissues' ability to derive nutrients from the media. Phenolic compound oxidation also results in generation of toxic derivatives which kill the plant tissues due to inhibition of enzyme activity [11]. Various studies have shown that dark culturing generally reduces tissue browning and improves plant proliferation [12], [13]. In addition, incorporation of antioxidants in culture medium was also shown to control tissue browning [14], [15], [16], [17]. Different vitamins, such as thiamine, also play a role in enhancing the plants' ability to tolerate oxidative stress [18].

Efforts to successfully micro propagate *M. balbisiana* were thus targeted at; increasing the concentration of cytokinin (BAP), reducing the light-mediated phenolic oxidation and reducing oxidative stress within the explants. This study therefore investigated the effect of BAP, various vitamins and photoperiod duration on proliferation of *M. balbisiana* with the aim of improving the micro-propagation protocol.

2. MATERIALS AND METHODS

2.1 Preparation of Explants

Fresh suckers (peepers) of wild *M. balbisiana* were used as explants. Roots were removed from the suckers and the base of corms reduced to 15 cm. These corm tissues were returned to the tissue preparation room. Separately, surface tissues of the corm and

pseudostem area were sliced away reducing the tissue to about 5-10 cm from the base and to about five pseudostem sheaths for each corm. Each tissue was then suspended in 95% ethanol for 5 min. After this time, the ethanol was discarded and the corms were submerged in warm water (42ºC) containing 10 drops/liter of liquid detergent for 25 min. Water was then discarded and the tissues were further sliced to remove the darkened surface tissue. They were then immersed in 95% ethanol for 5 min in 15% NaOCl (v/v) in sterile water containing 20 drops /liter of Tween 80 for 25 min. This solution was also discarded and the tissues were then rinsed 3-times in an equal volume of sterile water, after which they were ready for culturing on growth medium. Fresh banana corm tissues with apical meristems and a few surrounding leaf primordia were aseptically isolated and cultured on basic proliferation medium (Table 7) [19] with amendments.

2.2 Amendment of the basal MS with BAP and selected vitamins

All stock solutions for media preparation, except BAP and vitamins were autoclaved at 1.05 kg/cm^2, 121°C for 15 min before storage under refrigeration at 4ºC. Appropriate volume of a basal 1X proliferation medium was prepared by using appropriate volumes of stock solutions and adjusting the pH to 5.8 using 0.1N NaOH or 0.1N HCl. Gelrite was added as a gelling agent at a rate of 2.4g/l and the medium was autoclaved at 1.05 kg/cm^2, 121°C for 15 min.

To determine the effect of BAP, the basal MS proliferation media was supplemented with 5 mg/l, 7 mg/l, 9 mg/l and 10 mg/l of BAP by adding a corresponding volume from the stock. Similarly, the effect of nicotinic acid was determined by supplementing the basal MS proliferation medium with 0.5 mg/l, 0.7 mg/l, 0.9 mg/l and 1 mg/l of nicotinic acid. The effect of pyridoxine HCl was assessed by supplementing the basal MS proliferation medium with 0.5 mg/l, 0.7 mg/l, 0.9 mg/l and 1 mg/l of pyridoxine HCl. The effect of thiamine HCl was determined by supplementing the basal MS proliferation medium with 0.1 mg/l, 0.14 mg/l and 0.18 mg/l of thiamine HCl. The effect of ascorbic acid was also determined by supplementing the basal MS proliferation medium with 10 mg/l, 20 mg/l, 30 mg/l and 40 mg/l of ascorbic acid.

2.3 Tissue culturing and sub-culturing regimes

Using sterile blades, surface tissues for previously sterilized explant material were removed so that the tissue was reduced to about 2cm (transverse diameter at the thickest part of the corm area) having both a corm end and sheaths at the pseudostem end. This tissue was longitudinally sliced into two halves dissecting the meristem into two where each half was placed onto the proliferation media in baby food jars with the corm tissue end half way embedded into the media. The jars were sealed with cling film and incubated at 28ºC illuminated with white-fluorescent lamps. Tissues were transferred to fresh media every after 3 weeks.

2.4 Variation of the photoperiod duration

During the culturing and sub-culturing regimes, the duration of illumination was varied at 3 levels; 1) the normal 14 hr lighting and 10 hr darkness for all the 18 weeks of culture. 2) total darkness for the first nine weeks followed by the normal 14 hr lighting and10 hr darkness for the next 9 weeks and 3) total darkness for the first 12 weeks followed by normal 14 hr lighting and10 hr darkness for the next 6 weeks. Data was recorded on general appearance of tissues on media and the average number of shoots produced after 18 weeks of the culturing process. Generally four (4) explants were initiated for each treatment.

2.5 Data collection and analysis

The general appearance of explants throughout the 18 weeks of incubation was thus described and the number of resultant shoots/plantlets after 18 weeks of incubation was established. This data was subjected to statistical analysis using GENSTAT package. An ANOVA was conducted and the least significant difference between the mean number of shoots was determined and used to separate the means to determine those which were significantly different ($P<0.05$) at the different treatment combinations.

3. RESULTS

Proliferation of *M. balbisiana* varied (both in appearance and in number of resultant shoots) with type and concentration of vitamin and with photoperiod duration but not with increase in BAP concentration.

Table. 1: Number of shoots per corm of *M.balbisiana* after 18 weeks (wks) of culture on basal proliferation media supplemented with different concentrations of BAP for different weeks (wks) under different photoperiod/hours of lighting (hrs L).

Concentration of BAP (mg/l)	Mean number of shoots *		
	18 wks at 14 hrs L	9 wks at 0 hrs L followed by 9 wks at 14 hrs L	12 wks at 0 hrs L followed by 6 wks at 14 hrs L
5	0.00^{a}	1.00^{ab}	1.25^{b}
7	1.25^{b}	0.25^{ab}	0.00^{a}
9	1.25^{b}	0.25^{ab}	0.25^{ab}
10	1.25^{b}	0.00^{a}	0.25^{ab}
Lsd (5%)	1.069		
Fpr.	0.017		

*Means with the same letter are not significantly different

3.1 Tissue proliferation under different BAP concentrations and photoperiod duration.

Explants of *M. balbisiana* cultured under different concentrations of BAP and photoperiod duration often turned black together with the surrounding medium and eventually died (figure 1a). At the best performance, a single corm tissue (as starting explant material) resulted in only 1 shoot after 18 weeks of culture (table 1). Variability in the number of shoots was however high and the difference in the mean number of resultant shoot was significant ($P=0.017$). At a normal 14 hr photoperiod, there was an increase in the mean number of shoots from 0 to 1 with increase in BAP concentration from 5 to 7 mg/l.

Further increase in the concentration of BAP to 9 and 10 mg/l under similar photoperiod duration did not improve on the number of shoots. Similarly, the mean number of shoots increased from 0 to 1 at 5 mg/l BAP, when tissues were subjected to dark culturing for the first 9 and 12 weeks followed by a 14 hr photoperiod in the later stages of the culturing process. On the contrary, increasing the BAP concentration to 7, 9 or 10 mg/l together with culturing under dark condition for the first 9 and 12 weeks followed by a 14 hr photoperiod prevented tissue proliferation. This finding suggests that the concentration of BAP combined with incubation of tissues in the dark for 9 or more weeks are not the limiting factors and conditions to proliferation of *M. balbisiana* on basal MS medium.

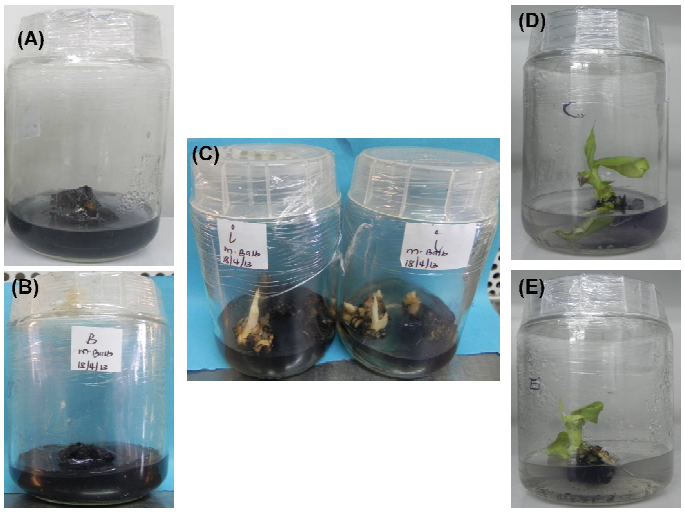

Fig. 1: Explant tissues of *M. balbisiana* (A) on basal MS proliferation medium at a 14hr photoperiod after 6 weeks, (B)on basal MS medium supplemented with 0.18mg/l thiamine HCl at 14hr photoperiod after 9 weeks (C) on basal MS proliferation medium supplemented with 0.18 mg/l thiamine HCl at 0 hr photoperiod after 9 weeks, (D) on Basal MS medium supplemented with 20mg/l ascorbic acid at a 0hr photoperiod for 9 weeks followed by a 14hr photoperiod for another 9 weeks and (E) on Basal MS medium supplemented with 0.18mg/l Thiamine HCl at a 0hr photoperiod for 9 weeks followed by a 14hr photoperiod for another 9 weeks.

Table. 2: Number of shoots per corm of *M. balbisiana* after 18 weeks (wks) of culture on basal proliferation media supplemented with different concentrations of Ascorbic acid for different weeks(wks) under different photoperiod/hours of lighting (hrs L).

Ascorbic acid concentration (mg/l)	Mean number of shoots*		
	18 wks at 14 hrs L	9 wks at 0 hrs L followed by 9 wks at 14 hrs L	12 wks at 0 hrs L followed by 6 wks at 14 hrs L
10	1.00[a]	1.25[a]	1.00[a]
20	1.00[a]	10.25[c]	5.50[b]
30	1.00[a]	6.75[b]	2.00[a]
40	1.33[a]	0.25[a]	1.00[a]
Lsd (5%)	2.125		
Fpr.	<0.001		

*Means with the same letter are not significantly different

3.2 Tissue proliferation under different ascorbic acid concentrations and photoperiod duration.

Generally, increase in the concentration of ascorbic acid above 10 mg/l in basal MS medium improved the proliferation of *M. balbisiana* to a significant level, resulting in 10 shoots per corm at the best performance, but only when explants were cultured in dark for the first 9 weeks (Table 3). Variability in the number of shoots was however high and the difference in the mean number of resultant shoot was significant (P<0.001). At a 14 hr photoperiod, a single corm gave only one shoot even when the ascorbic acid concentration was increased to 20, 30 and 40 mg/l. Under these concentrations most explants often blackened and died. However when explants were incubated in the dark for the first 9 weeks, proliferation significantly increased from one shoot per corm at 10 mg/l ascorbic acid to 10 shoots per corm at 20 mg/l ascorbic acid. Under these conditions, despite exudation of compounds that turned the medium to black in the immediate vicinity of the explants, there were some shoots that grew. Further increase in ascorbic acid concentration to 30 and 40 mg/l significantly reduced tissue proliferation to 7 and 0 shoots per corm respectively under similar dark culturing condition. Most explants together with the culture medium turned black and died. Prolonged incubation of tissues in dark for the first 12 weeks reduced the mean number of resultant shoots and under these conditions, although elevation of the ascorbic acid concentration from 10 mg/l to 20 mg/l significantly increased the number of shoots to six per explants, it was still below expectation. Under these conditions, most tissues and the culture medium turned black and died. Further increase in ascorbic acid concentration to 30 and 40 mg/l under similar prolonged dark culturing conditions reduced the mean number of resultant shoots to 2 and 1 respectively as most stopped growing and died. This finding seems to suggest that ascorbic acid facilitates proliferation of *M. balbisiana* optimally at concentrations of 20 mg/l only if incubated in the dark for the first 9 weeks after initiation followed by a 14 hr photoperiod and are thus limiting factors and conditions on basal MS medium.

Table. 3: Number of shoots per corm of *M. balbisiana* after 18 weeks (wks) of culture on basal proliferation media supplemented with different concentrations of Thiamine HCl for different weeks (wks) under different photoperiod/hours of lighting (hrs L).

Concentration of Thiamine HCl (mg/l)	Mean number of shoots*		
	18 wks at 14 hrs L	9 wks at 0 hrs L followed by 9 wks at 14 hrs L	12 wks at 0 hrs L followed by 6 wks at 14 hrs L
0.1	1.00ab	0.75a	1.00ab
0.14	2.25abc	7.25c	6.75bc
0.18	2.33abc	27.25d	21.50d
Lsd (5%)	5.934		
Fpr.	<0.001		

*Means with the same letter are not significantly different

3.3 Tissue proliferation under different thiamine HCl concentrations and photoperiod duration

Elevation of the concentration of thiamine HCl above the normal 0.1 mg/l commonly in basal MS medium combined with dark culturing improved the general proliferation of *M. balbisiana* in-vitro resulting in 27 shoots per corm under the best performance (Table 4). While variation in the mean number of resultant shoots per corm at different concentrations of thiamine HCl and photoperiod duration was high, the mean difference between respective means was highly significant (P<0.001). At 14 hr photoperiod, elevation of the concentration of thiamine HCl up to

0.18 mg/l only resulted in 2 shoots per corm as most tissues blackened and died. On incubation of tissues under dark conditions for the first 9 weeks, there was a significant increase in the number of resultant shoots from 1 at 0.1 mg/l Thiamine HCl to 7 and 27 shoots per corm at 0.14 mg/l and 0.18 mg/l thiamine HCl concentrations respectively, though tissues always turned pale due to lack of light. At prolonged dark culturing (for the first 12 weeks), there was an insignificant drop in the resultant shoots per corm to 6 at 0.14 mg/l thiamine HCl and to 22 at 0.18 mg/l thiamine HCl. Black staining of the medium was less intense under dark conditions. This finding seems to suggest that thiamine HCl facilitates proliferation of *M. balbisiana* at high concentrations of 0.18 mg/l and that light facilitates blackening which in turn limit proliferation of *M. balbisiana* on basal MS medium.

Table. 4: Number of shoots per corm of *M. balbisiana* after 18 weeks (wks) of culture on basal proliferation media supplemented with different concentrations of Nicotinic acid for different weeks(wks) under different photoperiod/hours of lighting (hrs L).

Concentration of Nicotinic acid (mg/l)	Mean number of shoots*		
	18 wks at 14 hrs L	9 wks at 0 hrs L followed by 9 wks at14 hrs L	12 wks at 0 hrs L followed by 6 wks at 14 hrs L
0.5	1.00[a]	1.00[a]	1.00[a]
0.7	0.75[a]	1.00[a]	1.00[a]
0.9	0.25[a]	0.25[a]	1.00[a]
1	1.00[a]	0.00[a]	0.50[a]
Lsd (5%)	1.312		
Fpr.	0.749		

*Means with the same letter are not significantly different

3.4 Tissue proliferation under different nicotinic acid concentrations and photoperiod duration

Elevation of the concentration of nicotinic acid above the normal 0.5 mg/l commonly in basal MS medium to 0.7 mg/l, 0.9 mg/l and 1 mg/l did not improve proliferation of *M. balbisiana* regardless of variation of photoperiod duration (Table5). While the variation in the mean number of shoot per corm was high, the difference in their means was not significant (P=0.749). Only 1 shoot resulted from a single corm under best performance. Most explants tissues and the medium often turned black under lighting and died. This finding suggests that low concentration of Nicotinic acid combined with tissue exposure to lighting are not the limiting factors and conditions to proliferation of *M. balbisiana* on basal MS medium.

Table. 5: Number of shoots per corm of *M. balbisiana* after 18 weeks (wks) of culture on basal proliferation media supplemented with different concentrations of Pyridoxine HCl for different weeks (wks) under different photoperiod/hours of lighting (hrs L).

Pyridoxine HCl Concentration of (mg/l)	Mean number of shoots*		
	18 wks at 14 hrs L	9 wks at 0 hrs L followed by 9 wks at14 hrs L	12 wks at 0 hrs L followed by 6 wks at 14 hrs L
0.5	1.00[a]	0.75[a]	1.00[a]
0.7	3.50[b]	9.25[d]	7.00[c]
0.9	0.25[a]	0.25[a]	0.75[a]
1	1.00[a]	0.33[a]	1.00[a]
Lsd (5%)	1.444		
Cv%	46.3		
Fpr.	<0.001		

*Means with the same letter are not significantly different

3.5 Tissue proliferation under different pyridoxine HCl concentrations and photoperiod duration

Elevation of the concentration of pyridoxine HCl above the normal 0.5 mg/l in MS medium coupled with culturing in the dark improved proliferation of *M. balbisiana* resulting in 9 shoot per corm under best performance (Table 6). While variation in the mean number of resultant shoots per corm at different concentrations of pyridoxine HCl and photoperiod duration was high, the mean difference between respective means was highly significant (P<0.001).

At a 14 hr photoperiod, there was a significant improvement in proliferation when the concentration of pyridoxine HCl was elevated from 0.5 mg/l to 0.7 mg/l resulting in 4 shoots per corm. Further increase in the concentration of pyridoxine HCl to 0.9 mg/l and 1 mg/l significantly reduced proliferation resulting in 1shoot per corm. Dark culturing (either for the first 9 or 12 weeks) at 0.5 mg/l pyridoxine HCl did not improve proliferation. However when the concentration of pyridoxine HCl was elevated to 0.7 mg/l under similar dark culturing conditions (for the first 9 and 12 weeks), proliferation significantly improved resulting in 9 and 7 shoots per corm respectively. Further elevation of the concentration of pyridoxine HCl to 0.9 mg/l and 1 mg/l under dark condition (either for the first 9 or 12 weeks) significantly reduced proliferation resulting in 1 shoot per corm. This finding seems to suggest that pyridoxine HCl facilitates proliferation of *M. balbisiana* optimally at concentrations of 0.7 mg/l and that this can be enhanced if tissues are incubated in the dark for the first 9 weeks after initiation followed by a 14 hr photoperiod. These are thus limiting factors and conditions to proliferation of *M. balbisiana* on basal MS medium.

Table. 6: Number of shoots per corm of *M.. balbisiana* after 18 weeks (wks) of culture on basal proliferation media supplemented with different concentrations of a combination of Nicotinic acid (NA), Pyridoxin HCl (PH), Thiamine HCl (TH) and Ascorbic acid (AA) for different weeks (wks) under different photoperiod/hours of lighting (hrs L).

Concentration of compound mixture	Mean number of shoots*		
	18 wks@ 14 hrs L	9 wks@ 0 hrs L followed by 9 wks@14 hrs L	12 wks@ 0 hrs L followed by 6 wks@ 14 hrs L
0.5NA+0.5PH+0.1TH+10AA	1.00a	0.25a	1.00a
0.7NA+0.7PH+0.14TH+20AA	30.00b	39.00d	36.00c
0.9NA+0.9PH+0.18TH+30AA	32.00b	39.00d	37.00cd
1NA+1PH+0.2TH+40AA	1.00a	1.00a	1.00a
Lsd (5%)	2.375		
Cv%	9.1		
Fpr.	< 0.001		

*Means with the same letter are not significantly different

3.6 Tissue proliferation under different combined vitamin concentrations and photoperiod duration

Generally, increasing the concentrations of vitamins (nicotinic acid, pyridoxin HCl, thiamine HCl and ascorbic acid in combination significantly (P< 0.001) improved proliferation of *M. balbisiana* resulting in 39 shoots per corm under best performance. At the normal 14 hr photoperiod, a combination of 0.7 mg/l nicotinic acid, 0.7 mg/l pyridoxine HCl, 0.14 mg/l thiamine HCl and 20 mg/l ascorbic acid resulted in 30 shoots per corm. At the

same photoperiod, further increase in the concentration of the vitamin combination to 0.9 mg/l Nicotinic acid, 0.9 mg/l pyridoxine HCl, 0.18 mg/l thiamine HCl and 30 mg/l ascorbic acid did not significantly improve proliferation resulting in 32 shoots per corm. Increased concentrations of the vitamin combination to 1 mg/l nicotinic acid, 1 mg/l pyridoxine HCl, 0.2 mg/l thiamine HCl and 40 mg/l ascorbic acid significantly reduced proliferation resulting in only 1 shoot per corm.

4. DISCUSSION

The failure in improvement of *M. balbisiana* shoot proliferation under elevations of 6-Benzylaminopurine (BAP) above 5 mg/l even under dark culturing condition contrasted with the findings of Kanchanapoom and Promsorn,[4] where increased concentration of BA improved the proliferation of a *M. balbisiana* variety 'Kluai Hin' (BBB genome). BAP is a contact growth regulator, effective at the site of contact. It is one of the plant growth regulators under the big group of cytokinins. These bring about tissue proliferation by binding to specific receptor molecules on cell surface or within the cytoplasm and therefore cell division and bud formation which begins with an asymmetric division of the target cell, several cells back from the tip. Further development of the target cell also requires the continuous presence of cytokinins [20]. The cytokinin leads to an increased uptake of calicium ions [21], binds to a regulating protein calmodulin resulting in calmodulin-calcium complex. This complex binds to and activates a number of enzymes including protein-kinase enzymes that add phosphorous to the serine or tyrosine hydroxyl group of proteins. This enzyme phosphorylation changes their activity. So the calcium calmodulin complex acts as a master switch, regulating alternative metabolic pathways within the cell, making calcium ions to act as secondary messengers, transforming the hormonal signal into a biochemical switch regulating the initial stages of bud formation [22]. Despite its role in improving proliferation, an increase in the BAP concentration in the growth medium did not improve *M. balbisiana* proliferation in this study suggesting that there was another limiting factor. Tissues and surrounding medium continued to blacken and died. As had been noted earlier that tissue browning was attributed in part to oxidative stress incurred at the cut surfaces of tissues or to oxidation of plant phenolic compounds in presence of light [10], [8], [9], [6], [5], [7], dark culturing to reduce on the light mediated oxidation did not improve proliferation. The oxidative stress tolerance capacity of medium as provided by ascorbic acid, thiamine HCl, pyridoxine HCl and nicotinic acid had to be investigated.

Elevation of the concentration of ascorbic acid from 10 mg/l to 20 mg/l improved the proliferation of *M. balbisiana*. Ascorbic acid is an established antioxidant. Cut plant tissues (including explants) always suffer oxidative stress arising from the mechanical injury leading to increased production of reactive oxygen species (including O^{-}_{2} and H_2O_2). To cause the antioxidant effect, ascorbic acid is oxidized by the generated reactive oxygen species to monodehydroascorbate (MDHA) radical which through a series of enzyme driven reactions, subsequently disproportionate to form ascorbic acid and dehydroascorbate [23]. While in other banana cultivars (especially the EAHB) 10 mg/l of ascorbic acid is normally sufficient to bring about an antioxidant effect, our finding showed that *M. balbisiana* has a higher requirement for this antioxidant. This implies that at injury, the oxidative stress effect in this genotype is stronger requiring a stronger anti-oxidation response and this was partially achieved through elevation of the concentration of exogenous Ascorbic acid. However, concentrations above 20 mg/l negatively affected proliferation. This is especially true because, excess ascorbic acid in the presence of free metal ions has been reported to initiate free radical reactions, making it a potentially dangerous pro-oxidative compound too. Proliferation at 20 mg/l ascorbic acid was not good enough as it only yielded 10 shoots.

Similarly, elevation of the concentration of thiamine from 0.1 mg/l to 0.18 mg/l improved the proliferation of *M. balbisiana*. Thiamine has previously been reported to play important roles in enzyme catalysis and alleviation of stress in different organisms including bacteria, fungi, animals and plants [24], [25]. The mechanism by which thiamine helps the plants to tolerate oxidative stress is not yet well understood but it has been reported to cause a reduction in the production of reactive oxygen species [18]. The culturing procedure that involved mechanical injury to the explants during in-vitro propagation of *M. balbisiana* inevitably induced oxidative stress to the explants tissues. The role of elevated concentration of thiamine in the increased proliferation of *M. balbisiana* is therefore likely to lie in its ability to inhibit oxidative stress reactions that would kill plant tissues, and this allowed the tissues to proliferate. The difference in the number of shoot generated under thiamine amendment in comparison to ascorbic acid amendment suggests that thiamin could be superior to ascorbic acid in alleviating the effects of oxidative stress in *M. balbisiana* during the culturing process.

In tissue culture reactions, nicotinic acid has been reported to increase embryogenesis in some plants [26]. In addition, this compound (a precursor for NAD and NADP) has been implicated in cellular redox reactions [27], [28] for which it would be expected to facilitate oxidative stress tolerance to cut plant tissues in the culture medium leading to improved proliferation. Results from this study indicate otherwise, suggesting that increasing its concentration beyond the basal recommendation in MS medium is only wasteful. Just like other vitamins, nicotinic acid is synthesized in plant tissues and only none established tissues would require the exogenously applied vitamin.

Similarly, in addition to being a co-factor in enzymatic reactions, pyridoxine HCl has also been reported to have antioxidant properties and modulates active oxygen species in plants [29], [30], [31]. It has been shown to protect some plants from photo-oxidative stress [32]. It's not a surprise that our findings showed the most significant increase in proliferation of

M. balbisiana following elevation of pyridoxine HCl concentration from 0.5 mg/l to 0.7 mg/l even under 14 hr photoperiod.

As noted earlier, the culturing procedure that involved mechanical injury to the explants during in-vitro propagation of *M. balbisiana* inevitably induced oxidative stress to the explants tissues. The role of elevated concentration of pyridoxine in the increased proliferation of *M. balbisiana* therefore is most likely to also lie in its ability to inhibit oxidative stress reactions that would kill plant tissues, and this allowed the tissues to proliferate. At elevated levels however, pyridoxine limited proliferation. Reasons for this limited proliferation are not clearly understood but elevated levels of pyridoxine were shown to compromise oxidative stress induced plant defenses [30].

This could imply that the redox state of plant cells is highly compromised, leading to lowered physiological functioning of cells including reduced tissue proliferation. Light has previously been shown to aggravate oxidation and despite the roles of ascorbic acid and thiamine in alleviating the effect of oxidative stress they could only bring about significant proliferation under dark conditions. Results have shown that while dark culturing reduced blackening of the medium, this phenomenon could not be eliminated entirely as explants could only be prepared in presence of light for proper visibility.

Furthermore, despite dark culturing proliferation would not improve without antioxidants. There is thus a synergistic effect from both dark culturing and increase on the concentration of antioxidants although prolonged dark culturing and excessive use of antioxidants subsequently negatively affects proliferation resulting in a few pale shoots. While proportional increments in the concentrations of vitamin combinations brought about increased proliferation of *M. balbisiana*, it is not known how it would perform when the vitamins are only combined at their best performing individual concentrations. Never the less, this study has demonstrated the importance of increasing the concentration of ascorbic acid and thiamin as antioxidants together with reduction of light mediated oxidation through dark culturing to banana explants which normally blacken and die under normal MS proliferation medium. Laboratories often adopt specific protocols for in-vitro culturing of a wide range of banana cultivars yet this study has demonstrated that different banana cultivars respond differently to cultural manipulations and thus require specific culturing conditions for best proliferation. Many of such cultivars often suffer intense browning similar to that suffered by *M. balbisiana*. It is thus recommended that similar modifications as revealed in this study be evaluated for such cultivars in order to come up with best cultivar specific protocols that give better proliferation rates.

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