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RESEARCH ARTICLE

In vitro studies under different cytokinin and auxin levels for the regeneration of banana seedlings from meristmatic shoot tips

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Abstract

The effects of two cytokinin (BAP and Kinetin) at different concentration levels were investigated on the shoot proliferation and regeneration of one Banana cultivar OSH 39 (5395–1) at the tissue culture laboratory of National Centre for Genetic Resources and Biotechnology (NACGRAB), Ibadan, Nigeria in 2018. The shoot tips were cultured on Murashige and Skoog medium supplemented variously at different concentrations of BAP: 3:5mg /L, 4.5mg /L and 5.5mg /L; kinetin: 3.5mg /L, 4.5mg /L and 5.5mg /L. The highest shoot proliferation was observed in both Cytokinin at 5.5mg/L while shoot elongation or regeneration was observed in Kinetin at 3.5mg/L. In most cases, optimum result for regeneration and proliferation was observed at 4.5mg/L in both BAP and Kinetin.

Keywords: Cytokinin, auxin, regeneration, proliferation, shoot-tip, banana.

Introduction

The cultivated Banana and plantain belongs to the Eumusa section of Musaceae of the genus Musa. Bananas and plantation are one of the world's major fruit crops and staple food for millions of people. According to Vulysteke *et al.*, (1992), plantain and Bananas provide more than 25% carbohydrate for over 70 million people and it ranks fourth in terms of gross value after paddy, wheat, milk, this makes it the largest fruit crop in terms of production.

Forcing buds under field condition is always a tedious and time consuming task; thus, amassing of a sufficient amount of suitable planting material is slow. The planting materials also suffer from poor phytosanitary quality (Vulysteke, 1989). Therefore shoot tip culture has been increasingly used as an alternative to conventional plant material since 1985 (Sagi *et al.*, 1998). The advantages of in vitro propagation of bananas and plantains over traditional propagation cannot be over emphasized. The merits include: multiplication rate, Physiological uniformity, availability of disease-free material all time, rapid dissemination of new plant material around the world, uniformity of shoots, vigorous growth in the early growing stages compared to conventional materials. Tissue culture also plays important role in the distribution of germplasm, conservation, safe exchange of planting material and rapid propagation of newly selected hybrid cultivars.

Besides the effects of genotypes/cultivars on in vitro propagation, shoot proliferation and regeneration are influenced by the types cytokinin and their concentration. (Cronauer and Krikorian, 1984). Hence it is necessary to explore the possibility of increasing the micropropagation rates by reviewing the current level of cytokinins used and improving on it. Wong (1986) asserted that when growth medium is supplemented with 11.1µm BAP, each of the explants will produce an average of 2.4 shoots; however, increasing the BAP concentration to 22.2µm and 44.4µm produced 2.6 and 4.3 shoots per explants respectively.

NAA, IAA or IBA are most frequently used to auxins to induce root initiation in banana (Vulysteke, 1989). Rooting can also be initiated on basal medium without any growth hormones (Cronauer and Krikorian, 1984). Vulysteke, (1989) found that NAA (1µm) was more effective than IAA. The optimum IBA concentration was found to be 1µm by Vulysteke and De Langhe (1985).

Therefore, the main objective of this study was to: investigate the effect of various levels of two cytokinins i.e The Benzylaminopurine (BAP) and Kinetin on the shoot proliferation and Regeneration of banana in vitro; with a view to improving on the current micro propagation rates.

Materials and methods

The study was carried out at the Tissue Culture Laboratory of National Center For Genetic Resources and Biotechnology (NACGRAB), Moor Plantation, Ibadan. The plant materials used were collected from the field trial plot of NACGRAB using full-grown banana variety OSH39 (5295-1). Suckers were removed from the mother plant with a spade.

In the laboratory, the superfluous tissues were removed from the collected suckers by trimming away the outer leaf sheaths, leaf bases and corm tissues until 1-2cm cubes enclosing the shoot apices were obtained. The cubes were then disinfected in 70% ethanol for 5minutes, followed by 7.5% and 5% NaOCl (Bleach) with few drops of tween-20 for 20 and 10 minutes respectively. They were later rinsed in three changes of sterile water. The culture medium used for this experiment was Murashige and Skoog (MS) medium (1962) to which auxins, cytokinins, vitamin stock, sucrose, inositol, ascorbic acid, activated charcoal and agar were added.

The medium was prepared by dissolving the appropriate chemicals as shown in the appendices in distilled water; the PH of the solution was adjusted to 5.7 with 01M NaOH and 0.1M HCl. Agar was added and the solution was heated to dissolve the agar. Before cooling, 15 millilitres of the medium was dispensed into culture tubes (sigma tubes) using a dispenser and then capped. The media was later sterilized by autoclaving at 121oC and a pressure of 1.05kg/cm2 for 15 minutes. The dissecting instruments used were placed in a 70% ethanol, and an alcohol lamp was used for repeated flaming. A sterile cube of tissue was placed in sterile Petri dish that has been calibrated. Holding the block steady with forceps, the superficial tissues that were exposed to bleach during disinfections were cut away from the sides of the cube. Using a dissecting microscope, the ensheating cones of leaf primordial were systematically removed by carefully cutting with a scalpel through the circular insertion of each primordial, thereby loosening it from the basal corm tissue. Holding the corm piece with the forceps, the loosened outer leaf primordial or initial was pushed away.

The conical shoot tip was reduced to the required size i.e. 5mm. At this stage, as much corm tissues as possible were trimmed from the base. The excised shoot tips were then inoculated into the culture medium in test tubes.

The cultures were labelled and transferred to the culture room, maintained at 25 ± 2oC and 4000 Lux of light. The cultures were transferred to fresh medium after three weeks during which they were screened for contamination (i.e. fungal and bacterial infections) and those contaminated were continually removed discarded.

The details of the media used are as follows: -
BAP1= 3.5mg/L (BAP) + 0.18mg/L (NAA)
BAP2=4.5mg/L (BAP) + 0.18mg/L (NAA)
BAP3= 5.5mg/L (BAP) + 0.18mg/L (NAA)
Kin1= 3.5mg/L (kinetin) + 0.18mg/L (NAA)
Kin2=4.5mg/L (kinetin) + 0.18mg/L (NAA)
Kin3= 5.5mg/L (kinetin) + 0.18mg/L (NAA)

Three weeks after inoculation, shoot tips had formed shoot clusters, which were subdivided into size groups. Individual shoot tips of comparable sizes were transferred unto fresh medium of various cytokinin levels. Data were taken on number of buds, roots, leaves per plantlet and plantlet height at three-week intervals after subculture. The data were taken for four successive three-week culture cycles. The data were analyzed using Analysis of Variance (ANOVA). The means were separated using the Duncans Multiple Range Test (DMRT) at 0.05 and 0.01 probability levels.

Results and discussion

Right from week three, the cytokinins differed significantly ($P < 0.05$) with respect to plantlet height. However, the type of cytokinin, treatment levels and their interactions did not show any significant effect on plantlet height and bud number (Table 1). From Table 2, the significant effects of the cytokinins as manifested in bud development six weeks in vitro ($P< 0.05$) was presented. The interactive effects of cytokinin and treatment levels were observed to be significant also. Whereas the treatment level was found to have significant effect on the plantlet height and bud number. Cytokinin effect differed significantly with respect to bud number ($P < 0.05$) and root number ($P < 0.01$) at the 9^{th} week. Treatment levels differed significantly with respect to plantlet height ($P < 0.01$) and bud number ($P < 0.05$). Also the interactive effect of cytokinin by treatment level was significant on bud number alone ($P < 0.05$). Moreover, at the 12^{th} week, significant cytokinin effects were observed for all plantlet characters at this final stage (Table 3 and 4).
Significant treatment effects were also observed for plantlet height, root number and leaf number. However the interactive effects of cytokinin by treatment levels were significant with respect to plantlet height, root number, and leaf number.

Table 1: Effect of cytokinin treatments on the growth of banana shoot tips *in vitro* (Subculture 1)

Source	Df	Plantlet height	Bud number	Root number	Leaf number
Cytokinin	1	0.308^{*}	0.834^{ns}	-	-
Treatment level	2	0.144^{ns}	4.356^{ns}	-	-
Cytokinin x Treatment level	2	0.009^{ns}	0.306^{ns}	-	-
Error	4	0.074	1.991	-	-
Total	9			-	-

*, ** represent significant difference at 5% and 1% probability level

Table 2: Effect of cytokinin treatments on the growth of banana shoot tips *in vitro* (Subculture2)

Source	Df	Plantlet height	Bud number	Root number	Leaf number
Cytokinin	1	0.118^{ns}	9.275*	0.979^{ns}	-
Treatment level	2	0.029**	10.436*	0.362^{ns}	-
Cytokinin x Treatment level	2	0.066^{ns}	1.406*	0.249^{ns}	-
Error	4	0.154	3.517	0.307	-
Total	9				

*, ** represent significant difference at 5% and 1% probability levels, ns means not significant

Table 3: Effect of cytokinin treatments on the growth of banana shoot tips *in vitro* (Subculture 3)

Source	Df	Plantlet height	Bud number	Root number	Leaf number
Cytokinin	1	0.663^{ns}	15.375*	13.356**	-
Treatment level	2	0.557**	11.090*	1.756^{ns}	-
Cytokinin x Treatment level	2	0.153^{ns}	2.507*	1.240^{ns}	-
Error	4	0.202	4.062	3.007	-
Total	9				

*, ** represent significant difference at 5% and 1% probability levels, ns means not significant

Table 4: Effect of cytokinin treatments on the growth of banana shoot tips *in vitro* (Subculture 4)

Source	Df	Plantlet height	Bud number	Root number	Leaf number
Cytokinin	1	0.007*	0.834*	53.984**	0.175*
Treatment level	2	66.431**	4.356^{ns}	7.264*	4.941**
Cytokinin x Treatment level	2	15.883**	0.306^{ns}	5.335*	1.541*
Error	4	0.872	1.991	1.48	0.212
Total	9				

*, ** represent significant difference at 5% and 1% probability levels, ns means not significant

Moreover, in Table 5, the interactive effects of cytokinins and treatment levels on the height of plantlets in the four-subculture periods were presented. Kinetin1 (3.5mg/L kinetin + 0.18mg/L NAA) gave the best results with progressive increase in mean plantlet height from week 3 to week 12. However, Plantlets increased in height progressively as period of observation increased irrespective of cytokinin level. In most cases BAP3 (5.5mg/L BAP + 0.18mg/L NAA) had the least effect on plantlet height.

Table 6 shows the interactive effects of cytokinin levels on number of buds in the four-subculture periods. BAP3 (5.5mg/L BAP + 0.18mg/L NAA) had the best increase effect on number of buds and increases as the number of weeks increased. This was followed by BAP2 (4.5mg/L BAP + 0.18mg/L NAA).

However, reduced number of buds was noticed in subculture 4 i.e. 12 weeks. This suggests that optimum result with respect to proliferation was attainable at subculture 3 (the result of the 9th week). The result indicated that root development did not commence until the 12th week i.e. subculture 4 stage under BAP treatment. However, kinetin1 (3.5mg/L kinetin + 0.18mg/L NAA) and kinetin2 (4.5mg/L kinetin + 0.18mg/L NAA) were able to initiate less than one banana plantlet root at the 6th week, which progressed to about 6 and 5 roots, respectively, at 12th week (subculture 4) from the commencement of the experiment. Thus, kinetin1 and kinetin2 plus 0.18mg/L of NAA were adequate for root development in banana plantlets *in vitro* (Table 7). It also reveals the rood development capacity of the kinetin1 and kinetin2 plus 0.18mg/L of NAA.

Result in Table 8 shows that in vitro leaf development would not start until 12th week (subculture 4) in banana irrespective of type and concentration of cytokinin used. However, kinetin1 (3.5mg/l Kinetin + 0.18mg/l NAA) gave the highest number of leaves on banana plantlets.

On the whole, higher bud number (proliferation rate) was observed at the highest concentration in (5.5mg/L) of the cytokinin BAP. This could be due to its large effects on cell division, and its ability to increase stem thickness, which leads to promotion of lateral buds. It slows down the root development at the same time and this is very likely because the wider leaves provide more nutrients to the plant and reduced the need for root mass. This agrees with the report of Arinaitwe *et al.,* (1999) on the effects of cytokinin / auxin combinations on proliferation of banana. It is also in agreement with the findings of Wong (1986), which showed that formation of multiple shoots or buds was promoted by supplementing the medium with relatively high concentration of cytokinin. The progressive increase in the number of buds as the subculture period increases suggest that subculturing promote bud proliferation.

However, the ability of kinetin to promote root, leaf development, and increased in plantlet height is probably due to promotion of root development in the presence of auxins and availability of nutrient to the plantlet through the roots. The few number of buds observed in kinetin is due to its inhibitory effect on lateral bud growth (Dore *et al.,* 1983). The reduction in the number of buds, leaf development, higher number of roots and increase in plantlet height at subculture 4 (week 12) suggest that the culture switched onto the regeneration stage and attained a full plantlet at subculture 4. It can also be deduced from these findings that banana culture attains its optimum bud proliferation at the 9th week while transplanting of the cultures or plantlets can commence from week 12.

Table 5: Interactive effect of cytokinin levels on plantlet height in four *In vitro* culture cycles

Level	Subculture 1	Subculture 2	Subculture 3	Subculture 4
BAP1	0.467[b]	1.017[a]	1.427[a]	5.938[b]
BAP2	0.270[c]	0.512[b]	0.758[b]	3.800[c]
BAP3	0.270[c]	0.506[b]	0.362[c]	1.570[f]
Kin1	0.670[a]	1.070[a]	1.690[a]	9.876[a]
Kin2	0.418[b]	0.652[b]	0.766[b]	3.240[d]
Kin3	0.520[b]	0.320[c]	0.838[b]	2.486[e]

Means followed by similar alphabets are not significantly difference at 5% probability level

Table 6: Interactive effect of cytokinin levels on number of buds in four *In vitro* culture cycles

Level	Subculture 1	Subculture 2	Subculture 3	Subculture 4
BAP1	1.800^{c}	2.667^{bc}	3.667^{c}	1.000^{cd}
BAP2	2.000^{bc}	4.600^{a}	5.000^{ab}	1.400^{bc}
BAP3	3.200^{a}	5.100^{a}	5.800^{a}	2.400^{a}
Kin1	1.600^{c}	1.200^{c}	2.000^{d}	0.600^{d}
Kin2	1.800^{c}	3.000^{b}	2.600^{c}	1.200^{c}
Kin3	2.600^{a}	4.000^{b}	4.400^{b}	1.800^{b}

Means followed by similar alphabets are not significantly difference at 5% probability level

Table 7: Interactive effect of cytokinin levels on root number in four *In vitro* culture cycles

Level	Subculture 1	Subculture 2	Subculture 3	Subculture 4
BAP1	-	-	-	2.667^{d}
BAP2	-	-	-	1.400^{e}
BAP3	-	-	-	2.400^{d}
Kin1	-	0.600b	0.800^{a}	6.200^{a}
Kin2	-	0.600b	1.000^{a}	5.200^{b}
Kin3	-	-	0.600^{b}	3.200^{c}

Means followed by similar alphabets are not significantly difference at 5% probability level

Table 8: Interactive effect of cytokinin levels on number of leaves in four *In vitro* culture cycles

Level	Subculture 1	Subculture 2	Subculture 3	Subculture 4
BAP1	-	-	-	1.600^{b}
BAP2	-	-	-	1.000^{d}
BAP3	-	-	-	0.800^{d}
Kin1	-	-	-	2.667^{a}
Kin2	-	-	-	1.800^{b}
Kin3	-	-	-	1.200^{c}

Means followed by similar alphabets are not significantly difference at 5% probability level

Conclusion

In most cases, higher bud numbers (proliferation rate) was observed in BAP while higher rooting, leaves and plantlet height (Regeneration rate) were observed in kinetin. Also, 5.5mg/L BAP + 0.18mg/L NAA concentration had the best performance for proliferation; 3.5mg/L Kinetin + 0.18mg/L NAA concentration showed lowest performance in proliferation while 4.5mg/L Kinetin + 0.18mg/L NAA concentration gave the intermediate performance.

The results of the experiment show that the higher the cytokinin concentration, the higher the proliferation rate and the lower the concentration, the higher the regeneration rate. Kinetin (3.5mg/L) can be used in regeneration medium because of its early rooting and elongation effects while BAP (5.5mg/L) is recommended for use in the proliferation medium because of its ability to induce multiple shoots.

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