ENHANCED *IN VITRO* PROPAGATION OF *MUSA ACCUMINATA* INDUCED BY HUMIC ACID FROM COAL EXTRACT AS COMPARED WITH COMMERCIALLY AVAILABLE HUMIC ACID PRODUCTS

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Abstract

Humic acids (also known as the black gold of agriculture) are complex molecules that exist naturally in humic matter found in soils and are an excellent natural and organic way to provide soil with a concentrated dose of essential nutrients, vitamins and trace elements. A source of humic acids is found in soft brown coal referred to as Leonardite. The Humic acid was extracted from Leonardite collected from Neyveli and it was estimated. The estimated Humic acid was used for the micropropagation of Grand Naine (Musa accuminata) at five different concentrations (0.1 – 0.5%) and compared with commercially available Keradix and Humic Rooting. The various trials were used to check the growth propagation of the Humic acid in full MS media, ¾ MS media, ½ MS media and ¼ MS media and comparison was made among the samples in full MS. After the micropropagation, the explants were selected for the initiation stage and the proliferation stage. The Rooting and Shooting stage were developed in the media with the Humic acid of all the samples against the control of various concentrations and the characteristics such as the Length, height, weight of the roots and shoots were studied and the best concentration for the growth of plants using Humic acid and the media were reported.

Keywords: *Grand Naine, Musa accuminata, MS media, Leonardite, Humic Rooting, Keradix, Micropropagation*

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1. INTRODUCTION

Humic substances are formed by the microbial degradation of dead plant matter, such as lignin. They are resistant to further biodegradation. Humic substances in soils and sediments can be divided into three main fractions: Humic acids, folic acids, and humin. The humic and fulvic acids are extracted as a colloidal sol from soil and other solid phase sources into a strongly basic aqueous solution of sodium hydroxide or potassium hydroxide [5]. Humic acids are precipitated from this solution by adjusting the pH to 1 with hydrochloric acid, leaving the fulvic acids in solution. This is the operational distinction between humic and fulvic acids. Humin is insoluble in dilute alkali. The alcohol-soluble portion of the humic fraction is, in general, named *ulmic acid*. So-called "gray humic acids" (GHA) are soluble in low-ionic-strength alkaline media; "brown humic acids" (BHA) are soluble in alkaline conditions independent of ionic strength; and fulvic acids (FA) are soluble independent of pH and ionic strength [14].

The functional groups that contribute most to surface charge and reactivity of humic substances are phenolic and carboxylic groups. Humic acids behave as mixtures of dibasic acids, with a pK value around 4 for protonation of carboxyl groups and around 8 for protonation of phenolate groups. There is considerable overall similarity among individual humic acids. For this reason, measured pK values for a given sample are average values relating to the constituent species [8]. The other important characteristic is charge density. The molecules may form a super molecule.

Secular structure held together by non-covalent forces, such as Van der Waals force, π-π, and CH-π bonds. Many Humic acids have two or more of these groups arranged so as to enable the formation of chelate complexes. The formation of (chelate) complexes is an important aspect of the biological role of humic acids in regulating bioavailability of metal ions [19].

Humic acid has direct effect on plant cell membrane which increases the permeability and make the mineral element move back & forth through the membrane, resulting in an increased transport of various mineral nutrient to site of metabolic need. When humic acid is applied to plant leaves, the chlorophyll content of leaves increases. Humic substances regulate plant growth hormones and inhibit the enzyme IAA oxidase there by hindering IAA destruction. They also provide many free radicals to plant cells that assist in exerting positive effect on seed germination, root initiation & plant growth. The best source of humic substances for fertilizer use is from linarite which is highly oxidized low grade lignite containing a relatively high concentration of smaller molecular units [15].

Increased N uptake by rough fescue (Fistula scabrella Torr.) in response to application of humic substances extracted from 3 soils, while P, K, Ca, Mg and Na uptake was unaffected [6]. Humic acid have long recognized that play a major role in producing morphological and physiological effects in plants [12] and [7]. It has been reported that humic acids are able to stimulate or inhibit plant growth depending on their differences in origin, nature and concentration. The application of humic substances to nutrient solution, to soil or sand has been documented and the results showed that they enhanced significant growth responses [20].

Tissue culture refers to the use of small pieces of plant tissue (explants) that are cultured in a nutrient medium under sterile conditions. By using the appropriate growing conditions for each explant type, plants can be induced rapidly in order to produce new shoots and by adding suitable hormones, new roots are induced. These plants can be divided usually at the shoot stage, to produce high numbers of new plantlets [16]. The new plants can be placed in soil and grown naturally. Also, healthy plants can be grown in the laboratory at any time. In vitro culture techniques of banana plants can produce thousands of plants in a relatively shorter time either using somatic embryo or apex explants which require different culture media for shoot multiplication and root differentiation [4]. Foliar sprays of HA promoted growth in many plants such as tomato, cotton and grape [1]. The present study is to study the effect of various concentrations of Humic acid in *Musa Acuminata*, a Cavendish variety often called as G-9 (Grand Naine) with different strengths of the Murashige and Skoog medium [11].

2. MATERIALS AND METHODS

2.1 Sample Collection

The coal sample (leonardite) was collected from Mines II of Neyveli lignite corporation, Neyveli. The commercially available Humic acid called Keradix was purchased from Akshaya Agro shop, Hosur and the Humic Rooting was collected from Genewin Biotech, Hosur.

2.2 Extraction of Humic Acid with Various Solvents

Humic acids were extracted from the resulting leonardite, using extraction methods that are capable of extracting humic acids 5 g of leonardite was extracted with 50 ml of each of the following (0.1 M NaOH, 0.1 M KOH, 0.1 M $Na_4P_2O_7$, 0.25 M NaOH, 0.25 M KOH, 0.25 M $Na_4P_2O_7$) and stirred for 1 min. The pH of the suspension was maintained at 13 by addition of NaOH (20%, w/v) and left undisturbed for three hours. The precipitate formation was eliminated by centrifuging the mixture at 3500 rpm for 15 min. The supernatant was acidified with 50 ml of 0.1 M HCl and stirred for 1 min. The pH of the suspension was adjusted to 1 by the addition of HCl (10%, w/v), and it was allowed to stand overnight. Both the aqueous fulvic acids and precipitated humic acids fraction were obtained by centrifuging at 3500 rpm for 15 min. The humic samples were dried at 60°C and the highest yield from each solvent extract was weighed [3].

2.3 KOH as Solvent:

10 gms of leonardite (Coal extracted Humic acid CHA) sample was weighed and ground. Fine particles were obtained by passing through a mesh sieve. Then the CHA sample was treated with 100 ml 0.1M KOH and mixed thoroughly, the complete dissolving of the Leonardite in KOH was ensured. Coal residue was then again treated with 5 ml of KOH. Water soluble salt of humic acid formed was filtered through a Whatmann No.42 filter paper to separate it from insolubles. 1 ml of concentrated hydrochloric acid was added to bring the pH < 2. The humic acid was precipitated in the bottom of the beaker. The precipitate thus obtained is Potassium Humate.

All the samples namely coal (Leonardite), Keradix and Humic Rooting were estimated for the Humic acid.

2.4 Estimation of % Humic Acid

The estimation of the percentage of Humic acid was elaborated by [18].

0.1g of humic acid sample was weighed, ground into a fine powder and sieved with 0.2 – 0.3 mm size mesh and dissolved in 10 ml of extraction buffer containing 0.2M NaOH, 0.0032 M DTPA (Diethylene triamine pentaacetic acid, ROLEX-Mumbai), 2% ethanol. The prepared aliquot of the sample was centrifuged to remove any particulates. The supernatant was used as the sample.1 ml of the sample was taken and mixed with 5 ml of water. The Absorbance was taken at 450 nm using Titan Biotech Humic acid as standard (50-300mg) [2].

2.5 Micropropagation of Grand Naine *(Musa Accuminata)*

MS Medium with full concentration, ¾ MS, ½ MS and ¼ MS were prepared with the following concentrations of hormones and humic acid. MS medium composition for one litre for all three stages are given in Table 3. Gelrite is used instead of agar in the concentration of 2.5g L^{-1} for good transparency

Explant Initiation Medium

The trials were carried out for the explant initiation medium against the control with full MS, ¾ M, ½ MS, ¼ MS. The pH maintained for the media was about 5.5-5.8 which was adjusted using suitable buffers.

Trial 1- Control-MS+3%Sucrose+IAA-3mg/l+NAA-1mg/l+Gelrite-2.5gm/l In the 1.1-1.5 trials, the MS media with various concentrations of 0.1- 0.5% were used.

Trial 2- Control- ¾ MS+3%Sucrose+IAA-3mg/l+NAA-1mg/l+Gelrite-2.5gm/l. In the 2.1-2.5 trials, the ¾ MS media with various concentrations of 0.1- 0.5% were used.

Trial 3- Control- ½ MS+3%Sucrose+IAA-3mg/l+NAA-1mg/l+Gelrite-2.5gm/l. In the 3.1-3.5 trials, the ½ MS media with various concentrations of 0.1- 0.5% were used.

Trial 4- Control- ¼ MS+3%Sucrose+IAA-3mg/l+NAA-1mg/l+Gelrite-2.5gm/l. Trial 4.1-4.5- In these trials, the ¼

MS media with various concentrations of 0.1- 0.5% were used.

Proliferation Medium: pH 5.5-5.8 was found as the optimum pH for the proliferation media which was adjusted using suitable buffers. The growth regulators were added in the proliferation medium.
Trial 5- Control-MS+3%Sucrose+6BAP-4.2mg/l+NAA-1mg/l+Gelrite-2.5gm/l. In the 5.1-5.5 trials, the MS media with various concentrations of 0.1- 0.5% were used.
Trial 6- Control- ¾ MS+3%Sucrose+6BAP-4.2mg/l+NAA-1mg/l+Gelrite-2.5gm/l. In the 6.1-6.5 trials, ¾ the MS media with various concentrations of 0.1- 0.5% were used.
Trial 7- Control- ½ MS+3%Sucrose+6BAP-4.2mg/l+NAA-1mg/l+Gelrite-2.5gm/l. In the7.1-7.5 trials, ½ MS media with various concentrations of 0.1- 0.5% were used.
Trial 8- Control- ¼ MS+3%Sucrose+6BAP-4.2mg/l+NAA-1mg/l+Gelrite-2.5gm/l. In the 8.1-8.5 trials, ¼ MS media with various concentrations of 0.1- 0.5% were used.

2.6 Shooting Medium

For the shooting medium, the optimum pH was 5.5-5.8 which was adjusted using suitable buffers.
Trial 9- Control-MS+3%Sucrose+Kinetin-10mg/l+NAA-1mg/l+Gelrite-2.5gm/l. In the 9.1-9.5 trials, the MS media with various concentrations of 0.1- 0.5% were used.
Trial 10- Control- ¾ MS+3%Sucrose+ Kinetin-10mg/l +NAA-1mg/l+Gelrite-2.5gm/l. In the10.1-10.5 trials, ¾ MS media with various concentrations of 0.1- 0.5% were used.
Trial 11- Control- ½ MS+3%Sucrose+ Kinetin-10mg/l +NAA-1mg/l+Gelrite-2.5gm/l. In the 11.1-11.5 trials, the ½ MS media with various concentrations of 0.1- 0.5% were used.
Trial 12- Control- ¼ MS+3%Sucrose+ Kinetin-10mg/l +NAA-1mg/l+Gelrite-2.5gm/l. In the 12.1 – 12.5 trials, ¼ MS media with various concentrations of 0.1- 0.5%

2.7 Rooting Medium

For the shooting medium, the optimum pH was 5.5-5.8 which was adjusted using suitable buffers
Trial 13- Control-MS+3%Sucrose+ Adenine sulphate-40mg/l+Kinetin-1mg/l+Gelrite-2.5gm/l. In the 13.1-13.5 trials, the MS media with various concentrations of 0.1-0.5% were used.
Trial 14- Control- ¾ MS+3%Sucrose+ Adenine sulphate-40mg/l+Kinetin-1mg/l + Gelrite-2.5gm/l. In the 14.1-14.5 trials, the ¾ MS media with various concentrations of 0.1-0.5% were used.
Trial 15- Control- ½ MS+3%Sucrose+ Adenine sulphate-40mg/l+Kinetin-1mg/l +Gelrite-2.5gm/l. In the 15.1-15.5 trials, the ½ MS media with various concentrations of 0.1-0.5% were used.
Trial 16- Control- ¼ MS+3%Sucrose+ Adenine sulphate-40mg/l+Kinetin-1mg/l +Gelrite-2.5gm/l. In the 16.1-16.5 trials, the ¼ MS media with various concentrations of 0.1-0.5% were used.

Medium for all the above trials were prepared and poured in sterilized Tissue culture bottles to a volume of 50 ml per culture bottle approximately, labeled and sterilized in the vertical autoclaves at 121º C at 15 PSI for 15-18 minutes in autoclave. The sterilized bottles were kept under observation for one week to check for the growth of contaminants.

Comparison of the Samples

The samples namely Coal (Leonardite) (CHA), Keradix (KHA), Humic Rooting (BHA) of various concentrations 0.1 – 0.5% were used for the Initiation, Rooting and shooting stages of *Musa accuminata* using full MS as the medium to compare the growth of the roots, shoots and the root's height and compared against the control.

2.8 Proliferation Stage

Trimming of the Explant buds were done at the top and the base was dissected exactly into two halves for all the samples namely CHA, BHA, KHA and compared with the control. They were then placed in the Proliferation medium and Trial bottles were labeled as GW04-NA-P, 0.1, 0.2, 0.3, 0.4 and 0.5 respectively and taken to incubation room [17].

Culture Conditions:

Humidity: 40-45%
Light: 16 [h d^{-1}]
Temperature: 25±2ºC.

Particulate count: class 1, 00,000 maintained by air handling unit (AHU).

Labeling of culture trays was done with operator code GW04 (MSP), date of inoculation, 51/3/11 code stage- MULTI and kept for observations.

2.9 Shooting Stage

At the Shooting stage, the multiplied shoots were trimmed at the top and the base was dissected into small cultures containing 2-4 multi shoots in it for all the samples namely BHA, CHA, KHA and compared against the control. The Trial bottles were labeled as GW04-NA-S, 0.1, 0.2, 0.3, 0.4 and 0.5 respectively and taken to incubation room [10].

Culture conditions:

Humidity: 35-40%
Light: 16 [h d^{-1}]
Temperature: 27±2ºC.

Particulate count: class 1, 00,000 maintained by air handling unit (AHU). Labeling of culture trays with operator code GW04 (MSP), date of inoculation, 1/6/12 (week/working day in the week/year) code stage- INITIATION were done and kept for inference. After 15 days of incubation, the observations were noted.

2.10 Rooting Stage

At this stage, the Shooting stage cultures were dissected with the base and individual shoot bearing culture bases were

placed in rooting medium bottles for all the samples namely BHA, CHA, KHA and compared against the control. Trial bottles are labeled as GW04-NA-R, 0.1, 0.2, 0.3, 0.4 and 0.5 respectively and taken to incubation room [9].

Culture Conditions:

Humidity: 35-55%
Light: 16 [h d^{-1}]
Temperature: 30±2ºC.
Particulate count: class 1, 00,000 maintained by air handling unit (AHU).

Labeling of the culture trays with operator code GW04 (MSP), date of inoculation-3/6/12 (week/working day in the week/year) code stages- INITIATION were done and kept for inference. After 15 days of incubation, the observations were noted.

Statistical Analysis

10 replicates were carried out for each treatment. After the 21 days of *in vitro* propagation, plant height, mass, number of roots, root length etc. Mean, SD and variance of the above trials were analyzed for the samples HA, CHA, KHA. The data were analyzed statistically by using analysis of variance (ANOVA) and Least Significant Difference (LSD) [13].

3. RESULTS

Quantification of Humic Acid

The extraction of humic acids from leonardite 5 g with 0.1 M KOH yielded 0.8813 g, 0.25 M K0H yielded 0.3312g, 0.1 M NaOH yielded 0.2216 g and 0.25 M of NaOH yielded 0.2566 g, 0.1 M $Na_4P_2O_7$ yielded 0.6273g and 0.25 M $Na_4P_2O_7$ yielded 0.6994g. Results of extractant that used for extracting humic acids from leornardite showed that the greatest yield of humic aicds was obtained in 0.1M KOH and the lowest yield in 0.1M NaOH. So KOH is selected as the extracting solvent for the humic acid extraction process.

Table -1: Estimation of Extracted Humic Acid

S.NO	CONTENTS	BLANK	STANDARD SOLUTION S1	S2	S3	S4	S5	TEST SOLUTION
1	Volume of standard solution (µl)	-	500	1000	1500	2000	2500	-
2	Concentration (µg)	-	500	1000	1500	2000	2500	-
3	Volume of Sample (µl)	-	-	-	-	-		1500
4	Volume of distilled water (ml)	3	2.5	2.0	1.5	1.0	1	1.5
5	Volume of 0.2 M NaOH + 0.003 M DTPA (ml)	2	2	2	2	2	2	2
6	Volume of ethanol (µl)	100	100	100	100	100	100	100
	Keep all the tubes in room temperature for 30 minutes							
7	Optical Density at 495nm	0.00	1.521	1.612	1.708	1.780	1.870	1.615

CALCULATION:
OD 1.615 corresponds to 1150 mg of humic acid
1.5 ml of sample contains = 1150 µg of humic acid
1 ml of sample contain 1000
= ---------- X 1150
1500
= 0.666 X 1150
= 766.66 µg/1000 µl
= 76.6% of humic acid
76.6% of Humic acid was derived from 10 gm of Leonardite from mines II of Neyveli yielded by dissolving the coal (Leonarddite) with 100 ml of 4% KOH. The standard Humic acid was tested against the sample. The concentration was reduced to 0.1% to 0.5% in the MS medium preparations

Table - 2: Comparison Of Samples For The Estimation Of Humic Acid

Final Ppm For Standards

STANDARDS (mg)	CONCENTRATION (ppm)	ABSORBANCE	SAMPLES	CONCENTRATION SAMPLE(ppm)	ABSORBANCE SAMPLE
50	50	0.8135	Coal (Leonardite) CHA	6.4703	0.3926
100	100	13645	Humic Rooting BHA	271.5272	3.5663
150	150	2.3203	Keradix KHA	307.7484	4.0000
200	200	2.9488			
250	250	3.2968			
300	300	3.7192			

Since the samples were of 70%, the standards concentration must be converted to 70% from 100%. The standard concentrations for 100% were 50, 100, 150, 200, 250, 300 mg respectively. For 70%, the standard concentrations were converted as 35, 70, 105, 140, 175, 210 mg respectively. The dilution factor for the estimation of Humic acid was 6 X. For 1 ml of the sample, they were diluted 5 times as the sample concentration was high. So, the Dilution factor was 6X (1 X + 5X). The concentration of the samples with dilution factor was mentioned in the above table.

Table 3: Effect Of HA in the Shooting Medium

COMPOSITION	MEAN SHOOT LENGTH IN CHA(cm)	MEAN SHOOT LENGTH IN BHA SAMPLE (cm)	MEAN SHOOT LENGTH IN KHA(cm)
MS-control	3.56±1.05	3.56±1.05	3.56±1.05
MS+0.1%HA	4.22±1.17	3.73±0.98	3.66±1.15
MS+0.2%HA	4.07±1.08	3.83±1.02	3.93±1.17
MS+0.3%HA	3.8±1.12	3.52±1.18	3.63±1.08
MS+0.4%HA	4.05±1.15	3.72±0.99	2.91±0.97
MS+0.5%HA	4.04±1.0	3.91±1.05	2.98±1.1

In the Table 3, the mean shoot length was higher in CHA when compared to the other samples. The maximum length obtained was in MS + 0.1% HA of 4.22 ±1.17.

In the Table 4, the root length after the Rooting stage was maximum in BHA sample than the other samples. The maximum length obtained was 6.52 ± 1.4. The maximum root height was maximum in CHA of 8.35 ± 0.3.

NOTE: The table indicates only trials put with full strength MS medium as the other strengths (¾ MS, ½ MS and ¼ MS) medium involved showed least response with Humic acid and its commercially available products.

Table -4: Effect Of HA in Rooting Medium

COMPOSITION	ROOT LENGTH IN CHA(cm)	PLANT HEIGHT (cm)	ROOT LENGTH IN BHA (cm)	PLANT HEIGHT (cm)	ROOT LENGTH IN KHA (cm)	PLANT HEIGHT (cm)
MS-control	5.18±1.1	6.1±0.3	5.18 ±1.1	6.1± 0.3	5.18 ±0.5	6.1 ±0.7
MS+ 0.1% HA	4.05±2.6	5.99±0.5	5.97 ±2.6	3.81 ±0.5	3.93 ±0.8	6.33±0.98
MS+ 0.2% HA	4.35±3.1	8.35±0.3	5.75 ±3.1	3.96 ±0.3	2.91 ±1.01	4.42±1.0
MS+ 0.3% HA	3.96±1.4	6.33±0.1	6.52 ±1.4	4.31 ±0.1	3.52 ±0.9	5.26±1.02
MS+ 0.4% HA	3.81±3.5	5.44±0.1	5.33 ±3.5	4.0± 0.1	4.29 ±0.4	5.4 ±0.94
MS+ 0.5% HA	4.73±2.5	6.22±0.1	6.2± 2.5	4.32 ±0.1	3.07 ±0.6	3.9 ±0.86

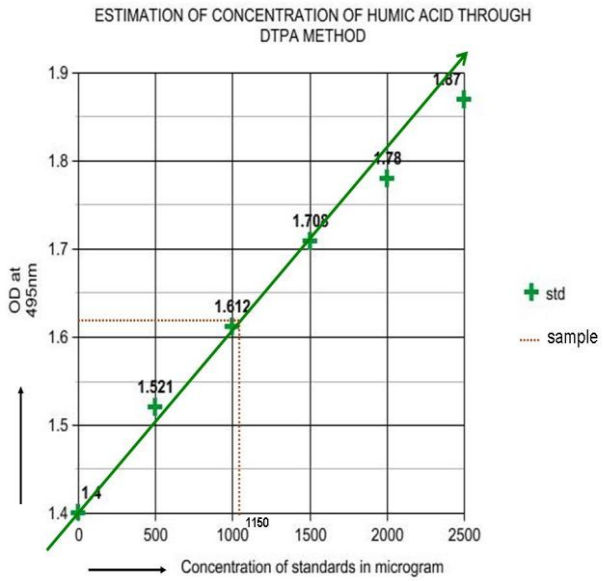
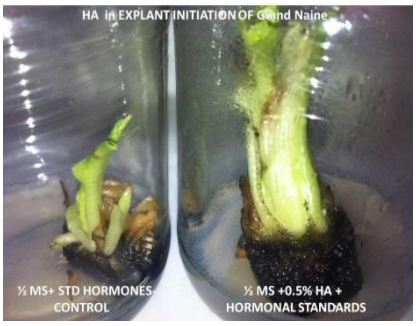


Fig 1: Estimation of Humic Acid in the Coal Sample before Comparison



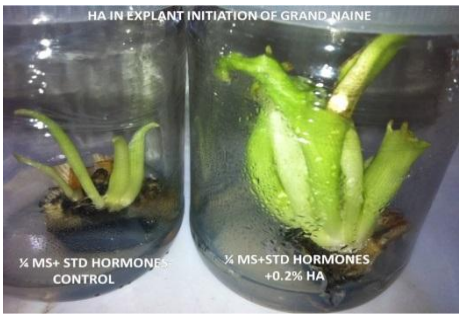


Fig 2: Explant initiation

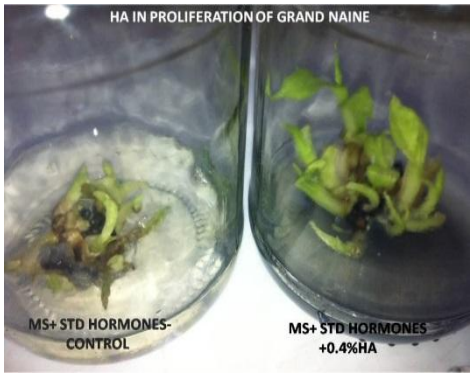


Fig 3: Proliferation stage

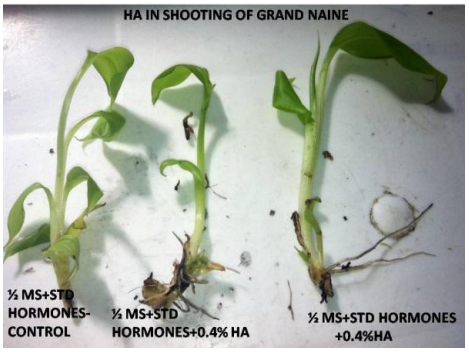


Fig 4: Shooting stage

Fig 5: Rooting stage

Fig 6: Fibrous roots in HA propagated plants

4. DISCUSSION

The differences in bud formation, multiplication ratio, shoot height and root morphology of control, Keradix and HA exposed plants were observed, but they were not statistically significant. The growth enhancement was also evaluated by the measurement of the fresh and dry weights of the leaves in 21-day-old control plants and the leaves in plants exposed to HA. The obtained results showed statistically insignificant differences in the mass of fresh and dry weight between the leaves of control and HA exposed plants

When explants were grown on MS medium with 0.1 to 0.5% of HA, ½ strength MS medium with 0.5% HA and ¼ MS medium with 0.2% HA with standard hormones showed highest bud formation percentage of about 78% and 3.12 g of fresh weight which is cause of excellent auxin concentration suspected in full strength MS medium with HA. The control seemed to have a high level of shoot and root length than others.

Among 3 cytokinins (BAP, Kinetin and TDZ), 6 BAP in combination with NAA is used in proliferation medium. In the trial, MS with 0.4% of HA gave highest multiplication ratio of about 5.03 while the controls varied from 1.00 to 2.5 ratio.

Shooting stage is performed with different MS strength with standard Kinetin and NAA combinations. HA is found to induce roots in all the multi and shooting trials where as MS, ¾ MS and ¼ MS medium augmented with kinetin and Adenine sulphate showed comparatively less response than ½ MS + 0.1 to 0.5% HA in rooting of *Musa accuminata*. Giant roots that are highly fibrous and good root length is observed after 21 days of incubation.

5. CONCLUSIONS

It was concluded that the coal (Leonardite) extraction is efficient using KOH as solvent and the micropropagation carried out using different concentrations was found to be higher in 0.4% HA which gave the higher multiplication ratio and the shoots were found to be induced in MS + 0.1% HA. Giant roots were observed at the rooting stage for the BHA sample with MS + 0.1 – 0.3% HA and the plant height was observed for the CHA sample with the MS + 0.2% HA. Commercially available Keradix seemed to have a slightly lesser effect when compared with CHA and BHA.

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