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STUDIES ON MICROPROPAGATION OF BANANA 'GRANDE NAINE'

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To achieve the micropropagation of banana, suckers with attendant rhizome explants (shoot tips from suckers and rhizome) were collected. They were thoroughly washed and cut into small pieces. The pieces were washed in labolene solution with few drops of Tween-20 and rinsed with distilled water. After surface sterilization with $HgCl_2$ solution, the explants were inoculated on solidified MS basal medium with benzyl adenine purine (BAP). The buds, which started growing, were transferred to multiplication media to obtain microshoots. The microshoots were rooted with 100 per cent efficiency, when transferred to a hormone free medium. The plantlets were acclimatized in two steps: for the first 4 weeks in culture room conditions and next 4 weeks in glass house conditions. The plantlets were grown for the initial 3 weeks in modified Knop's solution in hydroponics and then in the potting mixture containing soil, sand and FYM. The 100 per cent survival was recorded.

Key Words: Banana, Grande Naine, micropropagation, establishment, multiplication, hardening.

Traditionally, farmers cultivate 'Basrai' variety of Dwarf Cavendish group. The fruit is sweet, develops black spots on fruits upon ripening and has short shelf life. Basrai is very popular in India but it has virtually no international market acceptance (Chattopadhyay 1999). In order to exploit the vast international market, 'Grande Naine' has been introduced in India. It belongs to the AAA group and Cavendish (Giant Cavendish) sub group. This cultivar is popular in international trade and has a great potential for export market. It is less sweet compared to Basrai and has good keeping quality. It develops attractive yellowish green colour at maturity. It is internationally acceptable both as fresh fruit and in processed form due to high pulp to peel ratio (Anonymous 2005).

Banana is traditionally propagated by suckers. There are two types of suckers. 'Sword suckers' are thick at the base and narrow at neck with few linear sword shape leaves. 'Water suckers' are evenly thick at base and neck and bears broad leaves. Sword suckers of less than 0.9m heights are preferred for propagation (Chattopadhyay 1999, Rao 2005). However, conventional propagation of banana has limitations in respect of availability of large quantity of uniform and pathogen-free planting material of desired cultivar (Anonymous 2003). This problem can be solved by micropropagation, the clonal multiplication by plant tissue culture technique. Micro propagated plants show uniformity in flowering and harvest. Due to absence of seed production in most of the triploid bananas, the storage of germplasm in the form of *in vitro* culture has been an additional advantage for conservation (Anonymous 2003, Chattopadhyay 1999).

The objective of the present investigation was micropropagation of banana 'Grande Naine'. Its large-scale cultivation has not been attempted in India mainly because of non-availability of required number of propagules. There are scanty reports of tissue culture of 'Grande Naine' from India. In the present investigation a complete working protocol for the large-scale production of planting material of an international variety - 'Grande Naine', has been developed. All the experiments in this investigation were repeated thrice with 10 replicates in each treatment. The data in tables represent the means of the replicates in the

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three experiments.

There are three approaches of *in vitro* multiplication viz. forced axillary branching, organogenesis and somatic embryogenesis. Axillary / adventitious shoot formation is the most common method of micropropagation of banana. It consists of four stages viz. establishment of the plants *in vitro,* multiplication the shoots *in vitro*, rooting of shoots in vitro and transplantation of the *in vitro* raised plantlets.

MATERIALS AND METHODS

Stage I: Establishment

The banana explants i.e. the excised plant portions used to initiate the tissue culture, were collected from the campus of IFFCO, Phulpur, Allahabad. To obtain the shoot tip explants, 60-70 cm high sword sucker with attendant rhizome was lifted from the ground. Roots were removed. Rhizome was thorough washed under running tap water. About 2×3 cm sections containing shoot tips were excised. These sections were washed in 5% labolene solution with few drops of Tween-20 for 15 minutes and rinsed with distilled water. The plant material was further cut into about 5 mm explant carrying the shot tip, ensheathing leaf bases and 2-3 mm of basal rhizome tissue. Shoot apices, of 3-10 mm size, from the suckers were also used as explants.

Further surface sterilization of the explant was done under laminar airflow by the following two methods: (i) the explant was treated with 0.2% $HgCl_2$ solution for 5, 10 and 15 minutes; it was thoroughly washed with sterile distilled water; then given a 30 second dip in 70% ethanol and again rinsed in sterile distilled water; (ii) the explant was treated for 5,10 and 15 minutes with calcium hypochlorite [$Ca(OCl)_2$] with few drops of Tween-20; after treatment explant was washed with sterile distilled water; it was then given a 30 second dip in 70% ethanol and again rinsed in sterile distilled water.

The explant was then inoculated on solidified MS basal medium (0.75% agar at 5.8 pH) with 3% sucrose and 1mg / l BAP. (Observation was taken after 1 week for percent aseptic buds and after 3 weeks for percent bud breaks out of the aseptic buds (Table 1).

Stage II: Multiplication

Four experiments were conducted at this stage. In the first experiment the single shoots, produced at Stage I, were induced to produce multiple shoots on solidified MS basal medium with 3% sucrose and with different hormonal treatments. Observation was taken after 5 weeks for shoot multiplication (in fold i.e. number of shoots produced per initial shoot) and shoot length (Table 2).

The second experiment was conducted to study the effect of subculture duration (4 weeks, 5 weeks, 6 weeks, 7 weeks and 8 weeks) on the solidified MS basal with 2 mg/l BAP + 25 mg/l Ads and 0.1 mg/l IAA. Observation was taken for shoots multiplication and shoot length at the end of the specified subculture duration (Table 3).

In third experiment effectiveness of four different basal media for shoot multiplication were tested. Each of the four was solidified (0.75% agar at 5.8 pH) with 3% sugar, 2 mg/l BAP + 25 mg/l Ads + 0.1 mg/l IAA. Observation was taken after 6 weeks for shoot multiplication and shoot length (Table 4).

The fourth experiment was conducted to test the effectiveness of pH (5.2, 5.4, 5.6, 5.8 and 6.0). For this experiment borosilicate glassbead supported liquid MS basal medium with 3% sucrose, 2 mg/l BAP, 25 mg/l Ads and 0.1 mg/l IAA was used. Observation was recorded after 6 weeks for shoot multiplication and shoot length (Table 5).

Stage III: Rooting

Experiments on *in vitro* rooting were conducted on single shoots of more than 2 cm length, which were produced in Stage II. Smaller shoots were again passed through Stage II. Experiments were conducted on solidified MS basal with 3% sucrose and different hormonal treatment. Observation was recorded for rooting percentage, root length and shoot length after 4 weeks (Table 6).

Stage IV: Transplantation

Rooted shoots from 4-6 week old cultures were taken out from the solidified medium very carefully so that the roots were not damaged. The roots were washed very gently under running tap water to remove any adhering agar. The plantlets were put in test tubes containing modified Knop's solution (600mg/l $Ca(NO_3)_2.4H_2O$, 150mg/l $MgSO_4.7H_2O$, 150mg/l KNO_3 , 150mg/l KH_2PO_4 and MS microelements.

They were covered with transparent polythene bags with a small hole at the top and were kept in culture room conditions. The size of holes was increased after 3 days. The mouth of the polythene was opened after 1 week. The polythene bags were totally removed after 2 weeks. After 3 weeks the plantlets were transferred to small plastic cups / polythene bags containing autoclaved potting mix and the plantlets were kept in the culture room for another 1 week. They were irrigated once with Knop's solution and then with water as and when needed. After 4 weeks the plants were shifted to glasshouse were kept there for about 2-3 weeks and were then transferred to outside in shade.

Initially a number of potting mixes were tried viz. sand; soil; soil + vermicompost (3:1); soil + farm yard manure (3:1); and soil + sand + FYM (2:1:1). Later, only the mixture containing soil + sand + FYM (2:1:1) was used. Observation was taken for the effect of hydroponics and potting mixes on survival of plants (Table 7) and on root length (Table 8).

RESULTS AND DISCUSSION

Stage I: Establishment

The purpose of this stage is to successfully place an explant into aseptic culture by avoiding contamination and then to provide an in vitro environment that promotes stable shoot production.

This study has used shoot tips from suckers as well as from rhizome and the result was satisfactory. Shoot tips from rhizome or suckers have been the explant of choice (Hwang 1984, Vuylsteke 1989, Hamill 1993, Chattopadhyay 1999). In this study MS basal medium with 1 mg/l BAP was used for culture initiation. Banana cultures are generally initiated and grown on MS medium (George 1993/1996) with low concentration of only cytokinin (Gupta 1986) or both auxin and cytokinin (Banerjee and de Langhe 1985).

There are many reports of browning. To prevent browning, frequent transfers to fresh medium and/or addition of antioxidants (e.g. ascorbic acid) have been employed (Banerjee & de Langhe 1985, Gupta 1986). This study faced no noticeable problem from browning and therefore no efforts were made to prevent it.

In most banana micropropagation systems, semi-solid media are used. As a gelling agent agar is frequently used (Strosse *et al* 2004). This investigation has also used agar-solidified medium. However, there are reports of liquid media for banana (Alvard *et al* 1993, Bhagyalakshmi & Singh 1995).

This investigation encountered both bacterial as well as fungal infections in the culture. The fungal infections made their appearance in cultures 2-3 days after inoculation, but

bacterial infections as late as 1-2 weeks after inoculation. Three genera of fungi viz. *Aspergillus, Fusarium* and *Rhizopus* were observed as contaminants in cultures. The dark brown-black *Aspergillus* niger was most prevalent.

Table 1 Effect of different surface sterilization treatments on explants of banana 'Grande Naine'

No.	Treatment	Aseptic buds (%)	Bud break (%)
1	$HgCl_2$ - 5min	23.33	16.67 (71.42)
2	$HgCl_2$ - 10min	50.00	36.67 (73.33)
3	$HgCl_2$ - 15min	70.00	33.33 (47.62)
4	$Ca(OCl)_2$ - 5min	20.00	13.33 (66.67)
5	$Ca(OCl)_2$ - 10min	26.66	16.67 (62.50)
6	$Ca(OCl)_2$ - 15min	56.67	30.00 (52.94)

*The figures in parentheses show the percentage of bud break with respect to aseptic bud.

The effect of different surface sterilization treatments on explants of banana has been shown in Table 1. Although surface sterilization is achieved mostly by 70% alcohol and sodium or potassium hypochlorite with variation in procedure, concentration and duration (Cronauer and Krikorian 1985, Wong 1986, Hamill 1993), In this investigation, the use of calcium hypochlorite was not as good as mercuric chloride. The best result was recorded with 10-minute treatment of 0.2% mercuric chloride. It produced maximum bud break 36.67% (with respect to the number of aseptic buds, it was 73.33%). Although 15-minute treatment gives more aseptic buds, but because it might kill the explants more and also it gives fewer bud breaks.

Stage II: Multiplication

The purpose of this stage is to maintain the culture in a stable state and multiply the microshoots to the number required for rooting. In the present investigation, the multiplication of shoots took place simultaneously by two methods: few by axillary shoot formation and mostly by direct adventitious shoot formation. Okote and Schultz (1996) have also reported direct adventious bud formation. A few cultures also produced basal callus like structures. By careful observation, these cultures can be discarded. Ray (2001) has found a cluster of shoot arising from a common expanded mass of basal callus like tissue. However, Strosse *et al* (2004) have found new axillary and adventitious shoots arising directly from the explants.

Table 2 shows the effect of hormones on shoot multiplication. It was found that shoot multiplication was produced by BAP alone but it was enhanced when BAP was supplemented with Adenine Sulphate (AdS). Addition of IAA at low concentration in the medium containing BAP and AdS further increased the multiplication. The best multiplication (8.07 fold) was achieved with 2 mg/l BAP+25 mg/l AdS+0.1 mg/l IAA. Increase of BAP upto 3 mg/l increased shoot length but not the shoot multiplication. Further increase of BAP neither increased shoot multiplication nor the shoot length.

Table 2 Effect of different hormonal treatments on shoot multiplication of banana 'Grande Naine"

No.	Treatment (mg/l)	Shoots multiplication (fold)	Shoot length (cm)
1	1 BAP	3.26	2.12
2	1 BAP + 15 AdS	3.93	2.18
3	1 BAP + 25 AdS	5.80	2.20
4	1 BAP + 40 AdS	5.60	2.20
5	1 BAP + 25 AdS + 0.1 IAA	6.70	2.21
6	1 BAP + 25 AdS + 0.2 IAA	6.40	2.26
7	2 BAP + 25 AdS + 0.1 IAA	8.07	2.58
8	3 BAP + 25 AdS + 0.1 IAA	8.03	2.67
9	4 BAP + 25 AdS + 0.1 IAA	7.23	2.42

In addition to the nutrients, it is generally necessary to add one or more growth substances, hormones, to support growth and morphogenesis in cultures. However, the requirement for these substances varies with the tissue, and it is believed that it depends on their endogenous levels. In banana shoot multiplication is achieved by supplementing

the medium with relatively high concentrations of cytokinin alone or in combination with low concentration of auxin IAA (Banerjee & de Langhe 1985, Wong 1886).In this investigation, combination of two cytokinins, 2 mg/l BAP and 25 mg/l AdS and one auxin, 0.1 mg/l IAA has produced maximum multiplication of 8.07 fold.

The rate of multiplication depends both on cytokinin concentration and the genotype. Ray (2001) has reported 5-10 fold multiplication. Strosse *et al* (2004) reported that shoot tips of cultivars having only A genome produces 2-4 new shoots, whereas cultivars having one or two B genomes produce a cluster of many shoots and buds. In the present investigation,

Table 3 Effect of subculture duration on shoot multiplication of banana 'Grande Naine'

No.	Subculture duration	Shoots multiplication (fold)	Shoot length (cm)
1	4 Week	5.63	2.18
2	5 Week	7.90	2.60
3	6 Week	8.73	2.67
4	7 Week	8.70	2.73
5	8 Week	8.77	2.76

*The figures in parentheses show the percentage of bud break with respect to aseptic bud.

about 8-fold multiplication in 'Grande Naine', which contains only A genomes, is quite satisfactory.

Table 3 indicates that the shoot length increased with increasing subculture duration from 4-weeks to 8-weeks. The shoot multiplication also increased up to 6-week but remained almost constant at longer subculture durations. Further increase in subculture duration beyond 8 weeks caused drying and finally leading to death of shoots. Subculturing is generally required when the density of shoots become excessive or due to accumulation of toxic metabolites or exhaustion or drying out of the medium (George 1993). In the present investigation 6 weeks subculturing cycle was found to be ideal because no significant increase in shoot multiplication was observed even in 8-week cultures. Subculturing at 4-6 week interval has been reported by many workers (Ray 2001, Strosse *et al* 2004).

Table 4 Effect of basal media on shoot multiplication of banana 'Grande Naine'

No.	Basal medium	Shoots multiplication (fold)	Shoot length (cm)
1	MS	8.80	2.64
2	SH	6.83	2.54
3	LS	6.56	2.46
4	WPM	4.66	2.04

Table 4 shows the effect of the four types of basal media, keeping all other constituents constant. The maximum shoot yield and shoot length were found in MS (Murashige and Skoog 1962) and minimum in WPM (Lloyd and McCown 1980). SH (Schenk and Hilderbrandt 1972) medium was a little better than LS (Linsmaier and Skoog 1965) medium (6.56 fold, 2.46 cm). A medium is 'a formulation of inorganic salts and organic compounds for the plant culture'. Generally, a medium is designed for a particular plant by using a standard basal medium. MS medium is a 'high salt' basal medium with ammonium (20 mM) as well as nitrate (39 mM) ions. Perhaps banana prefers high concentration, especially of nitrogen, which is present in the MS

Table 5 Effect of pH on shoot multiplication of banana 'Grande Naine'

No.	pH of medium	Shoots multiplication (fold)	Shoot length (cm)
1	5.2	2.93	1.67
2	5.4	4.10	1.88
3	5.6	5.76	2.00
4	5.8	8.63	2.62
5	6.0	6.30	1.91

medium. This result is in agreement with other workers, who also have found MS the best

medium for banana (George 1996, Ganapati et al 1995,1999, Chattopadhyay 1999, Ray 2001, Kodym & Zapata-Arias 2001, Strosse et al 2004).

Table 5 shows that the best shoot multiplication as well as the maximum shoot length was achieved at pH 5.8. This is in agreement with Alvard *et al.* (1993). Other workers have used pH 5.7 (Ganapati *et al.* 1999) and 5.7±1 (Bhattacharya *et al.* 1994). Selecting an initial pH ensures the availability of nutrients and most rapid rate of culture growth (George 1993). The pH of the medium is usually adjusted between 5.0 and 6.0 before sterilization. In general, a pH higher than 6.0 gives a fairly hard medium and a pH below 5.0 does not allow satisfactory gelling of the agar

Table 6 Effect of different hormonal treatments on rooting of shoots of banana 'Grande Naine'

No.	Treatment (mg/l)	Rooting (%)	Root length (cm)	Shoot length (cm)
1	0.5 IAA	73.33	3.67	3.94
2	1.0 IAA	52.34	3.16	3.56
3	0.5 IAA + 5 AdS	56.67	2.86	3.35
4	0.5 IAA + 10 AdS	46.67	2.18	3.16
5	0.2 IAA	100.00	4.20	4.68

(Bhojwani & Rajdan 1996).

Stage III: Rooting

Table 6 shows the effect of hormone on rooting of microshoots. In this investigation, single shoots were transferred to the medium containing IAA. IAA induced more rooting at low concentration. Increasing IAA lowered the rooting. Addition of a mild cytokinin AdS also decreased rooting. IAA at 0.2 mg/l IAA produced 100% rooting and maximum root length. Generally, the shoot length increased on rooting medium. This is because ncreased growth of root in rooting medium also enhanced shoot growth because roots are able to absorb nutrition better from the medium and provide it to shoots.

Rooting may be obtained on the shoot proliferation medium, spontaneously, if kinetin is used (Anonymous 2003). But, shoots are generally rooted *in vitro* on rooting medium that contains reduced or omitted cytokinin, an increased auxin and often reduced inorganic salts (Ray 2001). Auxins, IAA, NAA or IBA are commonly included in the medium at between 0.1 and 2 mg/l (Strosse *et al.* 2004, Gupta 1986, Vuylsteke 1989). Cronauer and Krikorian (1984a,b, 1985) obtained rooting by removing cytokinin or both the hormones. Vuylsteke & de Langhe (1985) obtained rooting by reducing macronutrients to 1/2 strength.

Stage IV: Transplantation

This stage involves the shift from a heterotrophic (sugar-requiring) to an autotrophic (photosynthetic) condition and the acclimatization of the microplants to the outdoor environment. The plants in vitro are exposed to controlled growth conditions, induce structural and physiological abnormalities in the plants, rendering them unfit for survival in outdoor conditions. The two main deficiencies of in vitro grown plants are: (i) poor control of water loss, and (ii) heterotrophic mode of nutrition (Bhojwani & Rajdan 1996). Therefore, before these plants are transferred to the green house or field conditions, they are put under the process of acclimatization or hardening to gradually adapt or adjust physiologically to the new environment (Donnelly & Vidaver 1988, George 1993, Bhojwani & Rajdan 1996, Hartman *et al.* 1997).

Table 7 shows the effect of hydroponics and potting mixes on survival of the tissue-culture raised plants. After 3 weeks, 100% survival was achieved in hydroponic culture, where in-vitro-raised plantlets were transferred to modified Knop's solution. Next best survival was obtained with sand irrigated regularly with

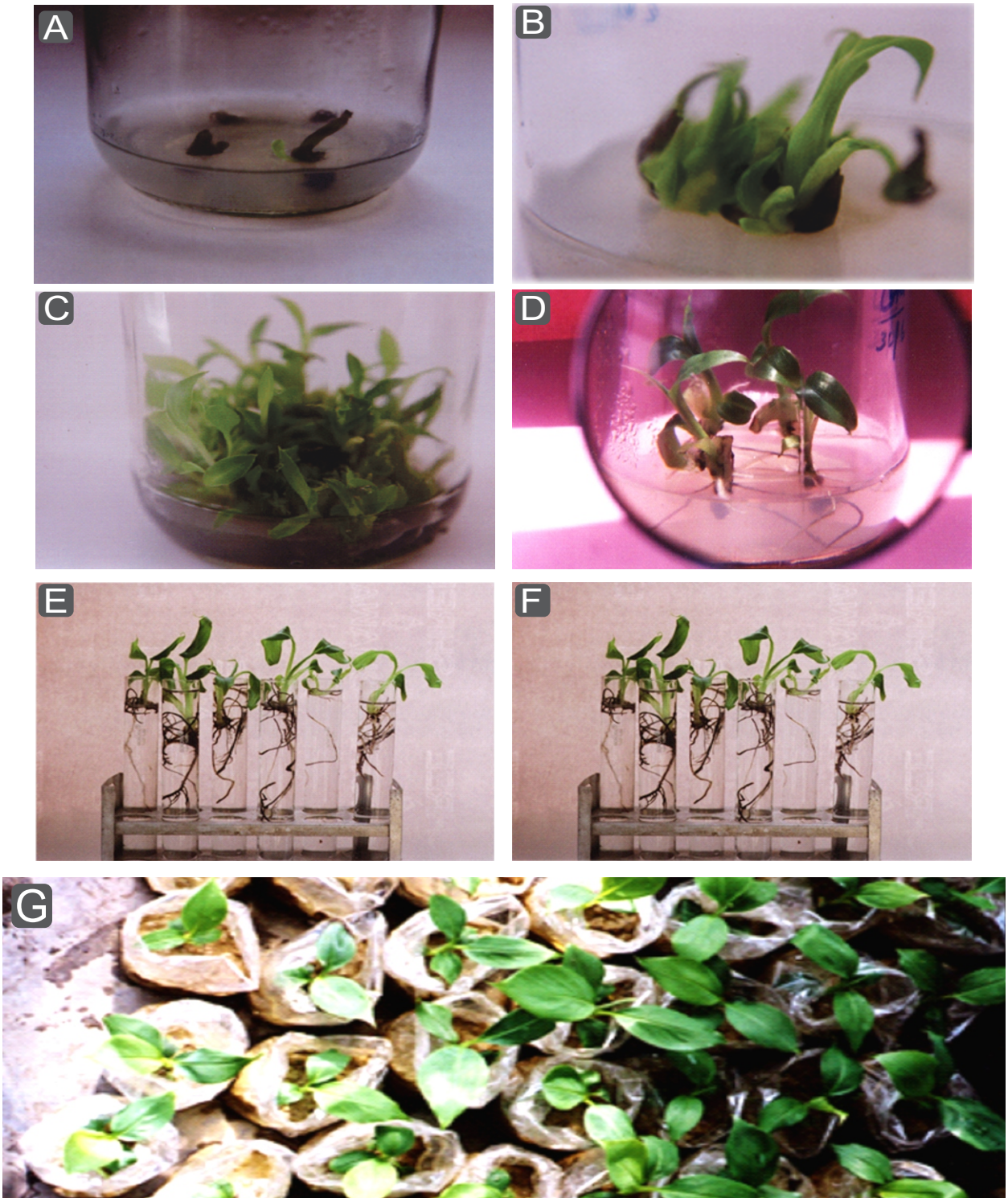

Plate 1: Micropropagation of banana ‘Grande Naine’. A Establishment stage. B-C Multiplication stage. D Rooting stage. E-G. Transplantation.

Knop's solution. After 8 weeks, 100% survival was obtained with 3-week old hydroponic plants transferred to the potting mixture

Table 7 Effect of hydroponics and potting mixes on survival of in vitro raised plantlets of banana 'Grande Naine'

No.	*Treatment	Survival after 3 weeks (%)	Survival after 8 weeks (%)
1	Soil	73.33	63.33
2	Sand	73.33	60.00
3	2 Soil + 1 Vermicompost (VC)	60.00	36.67
4	2 Soil + 1 Farm yard manure (FYM)	60.00	33.33
5	2 Soil + 1 Sand + 1 FYM	76.67	73.33
6	Knop's irrigated sand	86.67	80.00
7	Hydroponics and then in soil mix no. 5	100.00	100.00

*Treatments 1-5 were irrigated once with Knop's solution and then with water as and when required. Treatment 6 was irrigated regularly with Knop's solution.

containing soil, sand and FYM (2:1:1). Again the next best survival was obtained with sand irrigated regularly with Knop's solution. Potting mixes viz. soil with VC and soil with FYM produced poor survival.

Table 8 shows the effect of hydroponics and potting mixes on root length. The maximum root length was recorded in hydroponics but minimum in soil + FYM. Sand also gave satisfactory results with a moderate root length

The survival in different hardening medium can be correlated with the initial root growth.

Table 8 Effect of hydroponics and potting mixes on root growth of in vitro raised plantlets of banana 'Grande Naine'

No.	Treatment (mg/l)	Root length (cm)
1	Soil	5.87
2	Sand	8.16
3	2 Soil + 1 VC	3.14
4	2 soil + 1 FYM	2.68
5	Hydroponics	9.24

Because hydroponic medium provided least resistance to root, root growth was maximal and this helped the plantlets to survive better in later stages. The roots in Knop's irrigated sand also did not face much resistance due to large inter-particular spaces and root growth was good and thus survival percentage was also next only to that in hydroponics. Soil due to its compact nature did not allow the delicate *in vitro* formed roots to penetrate through. The low survival in soil + FYM and soil + VC was perhaps due to the inability of roots to absorb minerals from the complex organic matter like farmyard manure and vermicompost.

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