An efficient protocol for *in vitro* regeneration of Banana (*Musa spp*.) and acclimatization of Banana plants

ABSTRACT

Aim: The study was undertaken with a view to standardized a protocol for *in vitro* regeneration of Banana cv. Grand naine using shoot tip of sucker as an explant

Place and duration of study: The present study was conducted at Plant Tissue Culture Laboratory of K. K. Wagh College of Agricultural Biotechnology, Nashik, during the year 2022-2023.

Methodology: Inpresent study, explants sterilized with different sterilizing agents such as Tween-20 (1%), Bavistin (0.5-1%), Streptomycin sulphate (250 mg/L), Ascorbic acid (150 mg/L) + Citric acid (100 mg/L), $HgCl_2$ (0.1%) and 70% ethanol.Sterilized explants were cultured on Murashige and Skoog (MS) medium supplemented with different concentrations of plant growth hormones for shoot initiation BAP alone (1.5, 2.0, 4.0, 6.0 mg/L) and BAP (3.0 mg/L) in combination with IAA and IBA (2.0 mg/L), elongation BAP (3.0 mg/L) and NAA (1.0, 1.5, 2.0 mg/L); and rooting IAA (1.0, 1.5 mg/L) and IBA (1.0, 1.5 mg/L). Primary and secondary hardening was done in potting mixture containing autoclaved black soil: vermicompost: cocopeat (1:1:1) and garden black soil, cocopeat and red soil (1:1:1) respectively.

Results: In present investigation 1% Bavistin (fungicide) showed maximum respond to prevent fungal contamination. Highest shoot initiation (100%) was observed on a MS medium fortified with BAP (1.5 mg/L). Maximum shoot length (10.7 cm) was recorded on a MS medium supplemented with BAP (3.0 mg/L) + NAA (2.0 mg/L) + Activated charcoal.Maximum root initiation was observed on half strength MS medium supplemented with IAA (1.5 mg/L).*In vitro*regenerated plantlets hardened on the mixture of autoclaved black soil: vermicompost: cocopeat (1:1:1). After 14 weeks *in vitro* plantlets transferred in green house for acclimatization where, 80% survival rate was recorded.

Conclusion: Regeneration protocol was successfully standardized. Therefore, itcan be used for large scale propagation of healthy, disease- and virus-free planting material and *in vitro* propagation helps to meet higher demand of healthy planting material within shorter period.

Keywords: Banana, Grand naine, In vitro, Suckers, Activated charcoal

Introduction

Banana is a monocotyledonous, perennial, giant herb which belongs to the family musaceae[21].It is considered one of the most popular fresh fruits worldwide and all cultivars of Banana are nearly derived from *Musa acuminata* and *Musa balbisiana*. Name of the Banana originates from the Arabic word “banan”, which means the finger [7].Banana originated from the South East Asian region [9]. *Musa acuminata* (Malaysia) and *Musa balbisiana* (India) are the parent genome of todays cultivated Banana [15]. Banana is an important commercially growing fruit crop in India and Tamilnadu. Tamilnadu is one of the largest exporter and producer state in India [25].

The Banana plant is well known for its food, medicinal as well as industrial values [13]. Bananas are excellent source of the vitamins such as vitamin A, B6, C and D. It is medicinally important to reduced risk of high blood pressure, stroke, restore normal bowel

activity, energy booster, immunity booster, cholesterol lower in property [12]. Edible parts of the Banana are rich in nutritional elements, carbohydrates, phosphorus, potassium and minerals [11]. Banana is rich in nutritive value with high carbohydrates (22.2%), fibre (0.84%), protein (1.1%), water (75.7%), and fat (0.2%) [3]. Different parts of the Banana such as leaf, flower, pseudo stem, fruits used in medicine for the treatment of diseases like stomach disorder, arthritis, anaemia, kidneystone, neurodegenerative disease, etc.[24]. Bioactive compounds such as flavonoids, phenolic acid with anti-tumor, anti-depression, anti-bacterial, anti-ulcergenic properties present in Banana pulp. High quantity of potassium is present in Banana fruit and it also contains high iron content which is beneficial for anaemia patients and also helpful for controlling blood pressure [13].

Banana cv. 'Grand Naine' belongs to the important Cavendish subgroup and is valued for its excellent horticultural characteristics [19]. Grand naine is a high yielding cavendish variety, each bunch has 10 to 12 hands with 175-225 fruits. G-9 introduced to India from Israel [23]. More than 40% production of Grand naine takes place throughout the world [5]. Grand nainevariety possess high yielding and disease tolerance characters [13].

Propagation of Banana through vegetative propagation is easy but it takes longer duration for the large scale mass propagation of planting material and vegetative propagation serve as the potential source of transmission of fungal, bacterial and viral diseases which may affect on yield. Banana plant is highly susceptible to leaf spot and panama wilt diseases [6]. Vegetative method for the propagation of Banana using sucker is slow and time consuming process because a Banana plant produces only 5-20 suckers in its lifetime. In addition, spread of pests and diseases through conventional method is a major drawback for producing healthy planting material [27].

Plant tissue culture has a great potential to regenerate whole plant means totipotency ability it become important tool set for modern day Biotechnology [20]. Tissue culture plants have been reported to produce 39% higher yield as compare to the plants from sword suckers [8]. Plant tissue culture technique has great contribution as means of vegetative propagation of economically important species, especially for those difficult to propagate by conventional method such as seeds or cuttings [10]. The conventional method is not ideal for producing healthy planting material because they carry the disease and insect pest causing pathogen. Micropropagation through plant tissue culture technique is a best alternative for producing quality and healthy planting materials of Banana [25]. Plant tissue culture technology enable large scale production of uniform and ~~disease free~~disease-free planting material.

Micropropagation of Banana and plantain began in mid of 1970's. Plant tissue culture technique is potential to produce homogeneous plantlets [1]. Edible Bananas do not produce viable seeds so that, they are propagated through sword sucker therefore, there are chances to cause diseases this problem is overcome through the *in vitro* propagation of Banana which provides disease and ~~virus free~~virus-free healthy planting material to the farmers. Propagation of Banana through the conventional method is slow and time consuming and it is unable to meet high demand of healthy planting material therefore, this problem overcome through micropropagation technique. Tissue culture technique for Banana has high rate of multiplication, it requires small space to produce large number of plantlets within small space and it has higher survival rate. Therefore, keeping the market demand in mind and to overcome problems associated with conventional propagation method of Banana present study was conducted to establish a protocol for *in vitro* regeneration of Banana cv. Grand naine using shoot tip of sucker as an explant and to study the effect of plant growth hormones on the growth of explant.

2. MATERIAL AND METHODS

2.1 Explant collection and surface sterilization

The present study was carried out at plant tissue culture laboratory of K. K. Wagh College of Agricultural Biotechnology, Nashik-422003, during the year 2022-2023. Rhizome were collected from disease free healthy Banana field ~~inJalgaon~~ in Jalgaondistrict of Maharashtra state and the collected suckers were washed with running tap water to remove adhering soil. The scally leaves and roots removes carefully and suckers were trimmed about 4-5 cm in length Surface sterilized of trimmed suckers were done with 1% (v/v) Tween-20 for 10 minutes and rinsed with sterile distilled water followed by suckers soaked in a 1% (w/v)Bavistin (fungicide) solution for 20 minutes.Then, the explants were washed with sterile distilled water for 3-4 times and then, explants treated with 250 mg/L (w/v) streptomycin sulphate for 10 minutes to prevent bacterial contamination followed by explants washing with 2-3 times with sterile distilled water. The trimmed explants stored for 10 minutes in antioxidant solution containing 150 mg/L (w/v) ascorbic acid and 100 mg/L (w/v) citric acid. The pretreatment was done to overcome the problem of phenol exudation. Pretreated explants were surface sterilized with 0.1% mercuric chloride for 1 minute and 70% ethanol for 30 seconds. The explants were washed with 3-4 times with sterile distilled water to remove the traces of sterilants (Plate 1).

2.2 Culture condition and shoot initiation

The sterilized explants were inoculated on MS (Murashige and Skoog's, 1962) medium containing different concentrations of BAP (1.5, 2.0, 4.0 and 6 mg/L) either alone or in combination with same concentration of BAP (3.0 mg/L) and same concentration of IAA and IBA (2.0 mg/L) with 3% sucrose and 8 g/L agar. The inoculated culture bottles were kept in culture room at the temperature 25 ± 2 ^{O}C, relative humidity of 75-80%, 16/8 hr photoperiod and light intensity 1000-2000 lux for the growth of explant. The experiment was carried out in 3 replicationss with 5 explants for each treatment. Subculture was done in every 2 weeks of interval. ~~Observationswere recorded the~~ The shoot developed from the shoot initiation medium were transferred ontoshoot elongation medium containing ~~same concentration of~~ BAP (3.0 mg/L) with different ~~concentration~~ concentrationsof NAA (1.0, 1.5 and 2.0 mg/L) for 3-4 weeks. The experiment for each treatment was replicated 3 times.

Need subtitle for "shoot elongation"

Comment [MDE1]: How come BAP (1.5, 2.0, 4.0, and 6.0 mg/) combined with BAP (3.0 mg/L)? Please write clearer sentence.

Comment [MDE2]: How many shoot per container, per experimental unit?

2.3 Root induction and hardening

Well developed shoots were excised and inoculated on a half strength MS media fortified with IAA (1.0 and 1.5 mg/L) and IBA (1.0 and 1.5 mg/L) with 20 mg/L activated charcoal were added for 3-4 weeks. Rooted plantlets were successfully transferred to plastic pots containing autoclaved black soil, vermicompost and cocopeat (1:1:1) for primary hardening of 4 weeks. After 4 weeks primary hardened well developed plantlets transferred to large size pots containing garden black soil, cocopeat and red soil (1:1:1) for 50-60 days.

Comment [MDE3]: What are the criteria for well developed shoots? (shoot height, number of leaf, etc)

2.4 Data collection and analysis

Five traits viz., Number of days required for shoot initiation, shoot induction percentage, shoot length, root length and number of roots were recorded after specific interval of time.

Comment [MDE4]: Need rate of multiplication; or number of shoot per initial explant.

3. RESULTS AND DISCUSSION

3.1 Culture establishment

In the present investigation, explants were inoculated on MS basal medium ~~alone~~ (without PGRs) and MS medium supplemented with different types and concentration of BAP either alone or in combination with IAA and IBA (Table 1, plate 2). The observation ~~show~~ shows the establishment of culture on MS media with various concentrations of growth hormones. It was observed that the minimum time was required by the MS medium fortified with BAP (1.5 mg/L). The 100% shoot induction percentage was observed on MS media supplemented with BAP (1.5 mg/L) it requires shorter time (13 days) for culture establishment as compare to Ali *et al*., (2011) they obtained 88% shoot formation on BAP (1.5 mg/L) within 10.6 days.

Table 1. Effect of cytokinin and ~~auxinconcentrations~~ auxin concentrations on shoot initiation from shoot tip explant

~~Treatment code~~	Concentrations of PGR's (mg/L)	No. of explants inoculated	No.ofexplants responded	Shoot initiation (in days)	Shoot induction (%)
~~T0~~	MS (Control)	5	1	25	20
~~T1~~	**MS + BAP 1.5**	**5**	**5**	**13**	**100**
~~T2~~	MS + BAP 2.0	5	2	18	40
~~T3~~	MS + BAP 4.0	5	4	15	80
~~T5~~	MS + BAP 6.0	5	3	15	60
~~T6~~	MS + BAP 3.0 + IAA 2.0	5	4	16	80
~~T7~~	MS + BAP 3.0 + IBA 2.0	5	3	15	60

Comment [MDE5]: Why there are only 5 explants inoculated? Where are the 3 replications? Supposed to be 15 explants? (5 explants per experimental unit x 3 replications?)

Comment [MDE6]: For commercial use of banana micropropagation, the most important variable is the number of shoots per explant. Do you have these data? These data are important for the calculation of the multiplication rate and the efficiency of the protocol.

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3.2 Sub culturing and shoot elongation

The explants after 4-5 weeks were subculture by trimming off the blackened tissue. The shoots were elongated on MS media containing same concentration of BAP (3.0 mg/L) with different concentration of NAA (1.0, 1.5 and 2.0 mg/L) (Table 2). Among different combinations the MS medium containing BAP (3.0 mg/L) and NAA (2.0 mg/L) showed maximum shoot length (10.7 cm).

Table 2. Effect of BAP and NAA on elongation of shoot length

~~Treatment code~~	PGR's concentration (mg/L)	No. of leaves/ explant	Shoot length (cm)

~~T2~~	MS + BAP (3.0 mg/L) + NAA (1.0 mg/L) + A. C.	3	7.6
~~T3~~	MS + BAP (3.0 mg/L) + NAA (1.5 mg/L) + A. C.	4	10.2
~~T4~~	**MS + BAP (3.0 mg/L) + NAA (2.0 mg/L) + A. C.**	**5**	**10.7**

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*** A. C. – Activated Charcoal**

3.3 *In vitro* rooting

Root initiation was observed 3 weeks within the transfer of elongated shoots onto the half strength MS medium containing IAA and IBA (1.0, 1.5 mg/L) with 20 mg/L activated charcoal (Table 3). In the present study early rooting was observed when the MS basal medium supplemented with IAA.The maximum root length (10.5 cm) and 6-7 roots per shoot were observed on half strength MS medium supplemented with IAA (1.5 mg/L) after 2 weeks.Whereas the result are somewhat contradictorily with Magar N *et al*., (2019) foundoptimum rooting on half strength MS with IAA (1.0 mg/L).

Table 3. Effect of IAA and IBA on root regeneration from regenerated shoots of Banana

~~Treatment code~~	PGR's concentration (mg/L)	Root observation	No. of roots/ plant	Root length (cm)
~~T1~~	**½ MS + IAA (1.5 mg/L) + A. C.**	**Observed**	**7**	**10.5**
~~T2~~	½ MS + IAA (1.0 mg/L) + A. C.	Observed	5	9.2
~~T3~~	½ MS + IBA (1.0 mg/L) + A. C..	observed	4	7.6
~~T4~~	½ MS + IBA (1.5 mg/L) + A. C.	observed	4	4.8

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Comment [MDE7]: What these mean?

***A.C.- Activated Charcoal**

3.4 Hardening and acclimatization

Elongated and rooted plantlets were taken out from culture vessels and roots were carefully washed thoroughly with tap water to remove the Agar. *In vitro* regenerated roots treated with 0.1% (w/v) Bavistin for 2-3 minutes and washed with tap water and successfully transferred to plastic pot containing autoclaved black soil, vermicompost and cocopeat (1:1:1) and potting substrates were moistened with tap water where, 100% survivability of primary hardened plantlets showed. After 1 month Banana plantlets transferred for secondary hardening in large size pots containing garden black soil, cocopeat and red soil (1:1:1).). After 14weeks *in vitro* plantlets transferred in green house for acclimatization where, 80% survival rate was recorded.

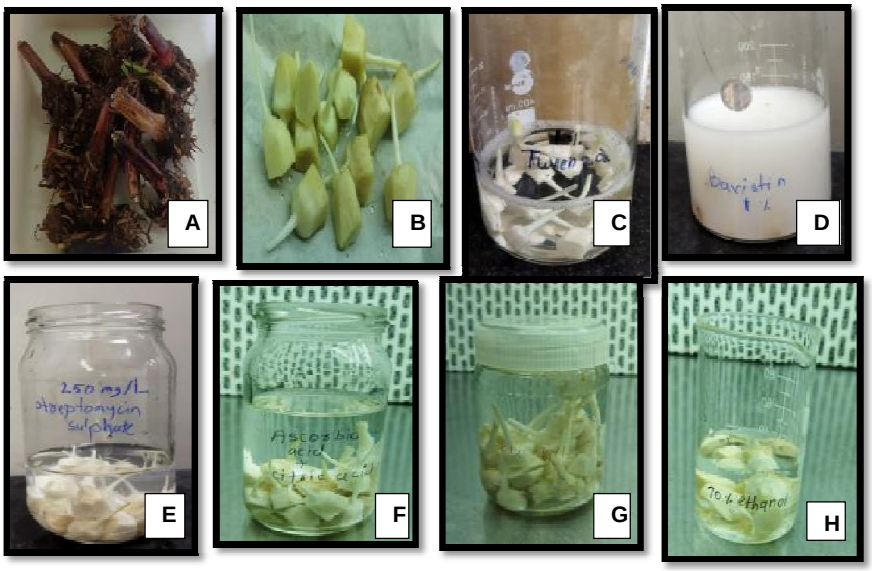

Plate 1. Sterilization of explants (Shoot tip) with different sterilizing agents

A) Suckers B) Shoot tip (explant) C) Treatment of explants with 1% Tween-20 solution D) Treatment of explants with 1% Bavistin solution E) Treatment of explants with 250 mg/L Streptomycin SulphateF) Treatment of explants with Ascorbic Acid (150 mg/L) + Citric Acid (100 mg/L) G) Treatment of explants with 0.1% Mercuric Chloride solution H) Treatment of explants with 70% Ethanol.


A
B
C
D
MS + IAA 1.5 mg/L
MS + IAA 1 mg/L
MS + IBA 1 mg/L
MS + IBA 1.5 mg/L
E

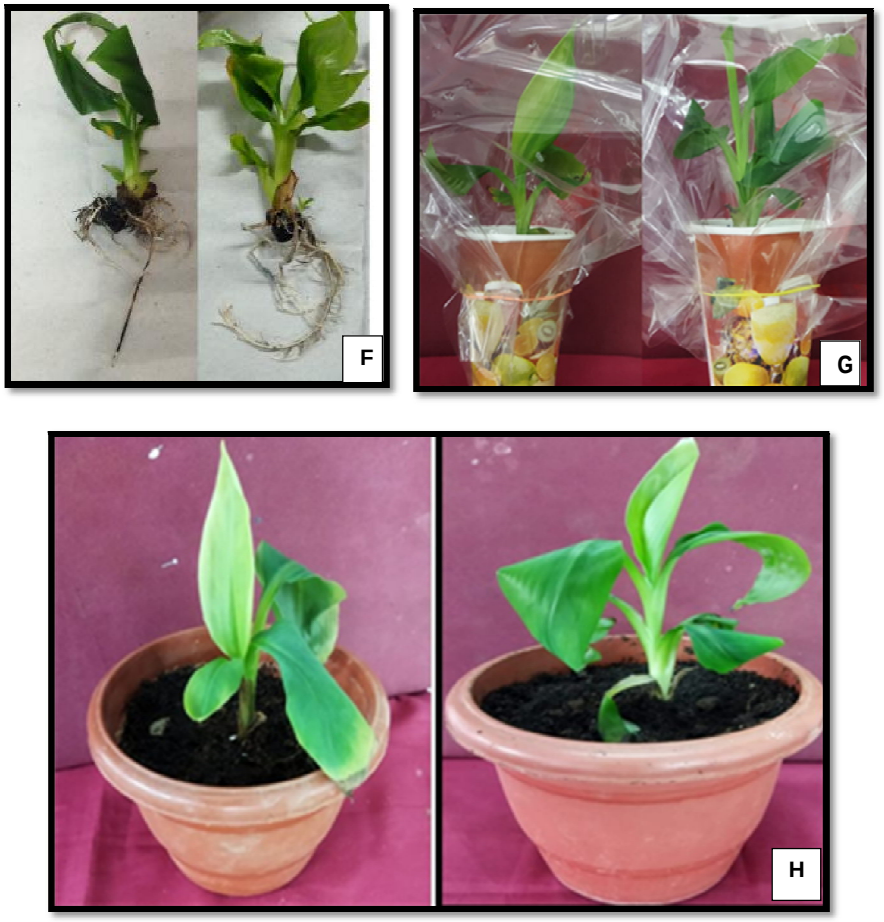

Plate 2.*In vitro* establishment of explant

A) Culture initiation B) Greening of culture C) Shoot induction on MS medium containing BAP (1.5, 2.0, 4.0, 6.0 mg/L) and BAP (3.0 mg/L) and IAA/ IBA (2.0 mg/L D) Shoot elongation on MS medium supplemented with BAP (3.0 mg/L) and NAA (1.0, 1.5, 2.0 mg/L) E) *In vitro* rooting on half strength MS medium fortified with IAA (1.0, 1.5 mg/L) and IBA (1.0, 1.5 mg/L) F) *In vitro* regenerated plantlets G) Primary hardened plantlets H) Secondary hardened plantlets.

4. CONCLUSION

The present study concluded that 1% Bavistin (fungicide) showed maximum respond to prevent fungal contamination. MS medium supplemented with BAP 1.5 mg/L showed highest response for shoot initiation. 4 to 5 leaves and elongated shoots about 10.7 cm were observed on MS medium supplemented with BAP (3.0 mg/L) + NAA (2.0 mg/L) + A. C. Half strength MS medium supplemented with IAA (1.5 mg/L) showed maximum response for the rooting. ~~Activated charcoal 20 mg/L used which prevents phenolic oxidation~~. During primary hardening 100% survivability observed whereas, 80% response of secondary plantlets were observed. Therefore, *in vitro* regeneration of Banana with various concentration of plant growth hormones can be used for large scale propagation of healthy, disease and virus free planting material and *in vitro* propagation helps to meet higher demand of healthy planting material within shorter period.

Comment [MDE8]: There is no data on sterilization treatment on the text, so you can not add this conclusion.

Comment [MDE9]: Can not be proved yet since there are no data of multiplication rate.

CONSENT

All the authors gave explicit consent to submit and that they obtained consent from the responsible authorities at the institute. It has not been submitted simultaneously for publication in any other journal.

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