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Study of *in vitro* regeneration technique through floral organ of banana cv. Grand Naine

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Abstract
Present investigation aimed to banana (CV. Grand Naine) optimize medium combination for callus induction and *in vitro* regeneration from male floral organ of banana (CV. Grand Naine). The study was conducted in Plant Biotechnology Centre, Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Dist. Ratnagiri (M.S) during the academic year 2020-2022. The various sized explants from male flower bud were transferred to MS medium containing different combination of PGRs. Among the various combination highest frequency of callus induction (86.00%) was observed in MS medium supplemental with MS + 2.5 mg/l 2, 4-D+0.5mg/l^{-1}+BAP+NAA. During development of callus different types of calli with variation in colour and texture were noted. Highest frequency of shoot regeneration from callus was reported in MS medium supplemented with 4.5 mg/l BAP. *In vitro* regenerated shoots successfully rooted (80%) in full strength MS medium fortified with 0.5 mg/l NAA + 1.5 mg/l IBA. *In vitro* regenerated plantlets were transferred to the pots with 80% success rate.

Keywords: Male flower, callus, MS medium, shoot regeneration

1. Introduction
The banana is a perennial monocotyledonous herb in the Zingiberales order. Musaceae is a family of plants. *Musa* and *Ensete* are the two genera that make up the Musaceae family. *Musa* consists of about 40 species and is distributed through India, New Guinea, Australia and Southeast Asia (Simmonds, 1962) [25]. Bananas are divided into two categories: dessert and cookery.

Banana includes three natural sugars: sucrose, fructose and glucose, as well as vitamins B6, Vit C, potassium, dietary fibres, which provide an immediate and considerable energy boost. The banana is known as the "Poor Man's Apple" since it is the cheapest of all the fruits farmed in the country by mean of nutritional and fruit value. Its fruits are commonly consumed raw as a dessert or cooked as a vegetable (plantains). Banana puree, breads, ice cream, chips, jam and candy are just a few of the ways bananas are consumed.

In terms of banana output, India leads the globe. India produced 33 million tonnes of bananas in 2021 (Statista Research Department, Oct 19, 2021), accounting for 26.43 percent of global banana production. China, Indonesia, Brazil and Ecuador are among the top five nations, accounting for 53.88% of the total. In 2020, global banana output is expected to reach 119 million tonnes. During the fiscal year 2021, India's available banana producing area was anticipated to be around 916 thousand hectares. Maharashtra is India's second-largest state by land and first-largest by production. Jalgaon is a prominent banana-growing district in Maharashtra, with 50,000 hectares under banana cultivation. However, the majority of bananas are produced by suckers.

Grand Naine (G-9) is a *Musa acuminate* cultivar that produces commercial Cavendish bananas. Because it is the principal product of Chiquita brands, it is also known as the Chiquita banana. The AAA genotype distinguishes this group of bananas from the others. This group is a triploid variation of the species *Musa acuminata*, as indicated by the AAA genotype. The AAA cultivar has 33 chromosomes, all of which yield seedless fruits by parthenocarpy. *Musa acuminata* is the scientific name for Grand Naine (AAA group). The edible fruit of the Grand Naine is produced by a huge inflorescence.

Planting materials that are not tissue cultured grown develop slower and yield smaller bunches. Fields developed using traditional planting material have a longer lifespan. In terms of producing homogeneous plants, the old approach is difficult, time consuming and inefficient (Banarjee and De Langhe, 1985) [4].

Banana production may be severely harmed by a variety of illnesses (Rahman *et al.*, 2004) [20], resulting in lower banana productivity and lower yields. In the past, a banana plant might only produce five to ten suckers every year, depending on the cultivar. As a result of the increased need for fresh seedlings at regular intervals, the demand for clean planting material has risen. The four most common viral infections that affect bananas are banana bunchy top virus (BBTV), cucumber mosaic virus (CMV), banana streak virus (BSV) and banana bract mosaic virus (BBMV). Furthermore, bananas are a popular host for Nematodes. Pests are also disseminated through non-quarantined planting material being transported.

The key to good banana cultivation is high-quality planting material. Tissue culture technique has been the backbone of mass-scale production of high-quality, disease-free planting materials, especially in a vegetatively propagated crop like banana. Micropropagation is a great way to get high-quality planting material at a minimal cost. This is a simple approach since it is easy to multiply, saves money on planting material and may produce 50-60 shoots per sucker in as little as 4-5 months. Micropropagation is the process of employing contemporary plant tissue culture technologies to multiply stock plant material to create a high number of offspring plants under aseptic conditions.

Because millions of plants can be grown from a single part of the plant within a year (Mantell *et al.*, 1985) [14] and plant multiplication can be done throughout the year, banana plantlets produced through micropropagation have been found to establish faster, stronger and healthier with a shorter production cycle and higher yield than conventional methods (Oritz and Vuylsteke, 1996) [18]. Tissue culture allows for the rapid production of huge numbers of banana plantlets. Other benefits of tissue cultured bananas include increased yield, uniformity, viral disease-free planting material and plants that are true to type (Robinson *et al.*, 1993) [21].

Male flower tissue culture technique produces an environment for disease-free planting material via mass multiplication and genetic enhancement, whether by direct organogenesis, indirect organogenesis, or somatic embryogenesis. The goal of this analysis is to highlight advancements in male floral tissue culture technologies that might be utilised to provide disease-free planting material and increase crop quality. Not only for genetic transformation, but also for the selection of useful somaclonal variants, *in vitro* regeneration via callus culture is critical (Vincent L and Anushma P L, 2018).

The current study was carried out with the objective, Standardization of *in vitro* regeneration technique through flower bud of Grand Naine (G-9).

Fig 1: Floral organ of Banana

2. Materials and Methods

2.1 Materials

2.1.1 Genotype

The present investigation was carried out at Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Ratnagiri. (M S). Study was carried out with popular *cv.* of Banana Grand naine. The experimental material (i.e., male flower buds.) was collected from department of Horticulture. Dr. Balasaheb Sawant Konkan Krishi Vidyapeeth, Dapoli, Ratnagiri.

2.1.2 Chemicals

Throughout the research, special care was taken to ensure that only high-purity chemicals were used. Growth hormones, vitamins, myoinositol, glycine, chelating agents (EDTA sodium salt) and other chemicals were obtained from Sigma (USA) and Hi- Media Chemical Company, respectively other chemicals, such as sucrose and agar, were obtained from Sysco Research Laboratories Pvt. Ltd., BDH and HiMedia Companies, respectively. The following are the various laboratory chemicals and biochemicals that were employed in the current study for media preparation and other purposes:

Chemicals for media preparation are Salts of macro and micro elements of analytical grade, Vitamins and amino acids, Sucrose as a carbon source, Myo-inositol, Agar-agar as a gelling agent, 0.1N HCl and 0.1N NaOH for pH adjustment, Plant growth substances, Auxin: IAA, 2,4-D, NAA, Cytokinin: 6-BAP, KIN.

2.1.3 Culture vessels and instruments

The borosilicate glassware utilised in this work was obtained from Borosil India Ltd. and the Corning Glass Company. Explants were cultured in test tubes, conical flasks and bottles.

Glassware used throughout the experiment, such as conical flasks, bottles and so on, was properly cleaned with laboratory detergent (tween-20), followed by adequate rinsing with running tap water to eliminate any detergent residues. After that, it was cleaned with distilled water and dried in a hot bed oven at 180°C for 2 hours. Plastic ware like as beakers and measuring cylinders, on the other hand, was wrapped in aluminium foil and sterilised in an autoclave at 121°C for 20 minutes at 15 lbs/inch2 pressure. The UV light in the laminar flow hood was turned on for 20 minutes before the procedure and turned off throughout it Prior to use, the laminar air flow system was turned on and the working surface was thoroughly cleaned with spirit. Forceps, scalpels and scissors were kept dipped in spirit under the laminar flow chamber and sterilised on the flame frequently throughout the procedure.

2.1.4 Experimental Conditions

In a laminar air flow chamber, all *in vitro* tests were performed aseptically. Experiments were carried out in a well-defined environment with a culture room kept at a constant temperature of 25± 2 °C and uniform light (1600 Lux) delivered by fluorescent bulbs (7200 K) throughout a 16/8-hour light/dark cycle.

2.1.5 Culture medium

Murashige and Skoog (1962) [29] produced a base medium that was supplemented with various concentrations and combinations of plant growth agents and antibiotics.

2.2 Methods

2.2.1 Preparation of Culture Media

After adding various Stocks to the MS basal medium (after bringing stock solutions to room temperature), the pH was

adjusted to 5.8 with 0.1N NaOH or 0.1N HCl. Final volume of medium was adjusted & during heating of medium on electrical heater 8 gm/l agar was added. After solubilization of medium it was dispensed into a suitable container. For autoclaving, a horizontal steam steriliser was used at 121°C and 1.5 kg/cm^2 pressure for 20 minutes. after autoclaving medium was stored in dark room & after 3 days it used.

2.2.2 Aseptic Techniques
For inoculation and subculturing of explants in culture bottles, normal sterilising protocols were used, as described by Street (1977). Explants were inoculated in a laminar airflow bench under aseptic conditions. All surgical instruments were dipped in alcohol, incinerated on a gas burner and cooled before use during the explant transfer.

2.2.3 Preparation of Explant for Culturing
2.2.3.1 Flower Bud
Musa acuminata cv. Grand naine flower buds were taken & disinfected with 70% alcohol & made little size of flower bud by removing of bract/leaves & little size of bud taken in culture plates. The male flower buds were reduced to 2-3 cm in length for culture in a sterile laminar hood and the tiny bracts and minute hands of the male flower buds were removed aseptically without damaging the apical dome and the male flower buds were inoculated on MS medium supplemented with different concentrations of 2,4-D plant growth regulators. From a single male flower, a total of 16-20 segments were recovered. After removing the inflorescence bud from the mother plants, cultures were started within 4 days.

Fig 2: Preparation of Explant for Culturing

2.2.4 Inoculation of explants for callus induction
Using an aseptic culture approach, flower buds were inoculated on callus induction MS medium supplemented with growth regulators. On the induction medium, the cultures were incubated in the culture room for up to 21 days in the dark. For *in vitro* callus induction of different explants on varied medium, combinations of growth regulators with variable doses of growth regulators were utilised. For callus induction, the following media combinations were employed.

Observations recorded
Frequency of callus induction, Days required for callus induction, Nature of callus: - friable/compact and hard.

2.2.4.1 Subculturing
Established cultures were transferred to the callus proliferation medium, MS media with 2.0 mg/l 2,4-D+ 0.5mg/l NAA+ 0.5mg/l BAP after 21 days of incubation and grown for three weeks (same light and temperature condition). Subculturing was carried out twice on the identical medium, light and temperature conditions.

Observations recorded
Weight of callus after 21 days

2.2.4.2 Regeneration of shoots from male flower bud induced callus
Subcultured callus was transferred to the following multiple shoot induction medium after 3-4 cycles. Bottle jar are incubated at 25±2 °C for 16 hours in the day and 8 hours in the dark.

2.2.4.3 Multiple shoots induction from *In vitro* shoot cultures
Regeneration shoots from callus were transferred to the multiple shoot induction media described below, each of which contained a different concentration of BAP.

Observation recorded
Number of shoot per callus mass**,** Per cent (%) response

2.2.4.4 Rooting
For root initiation from regenerated shoots, full MS medium was employed. This medium was treated with various concentrations of NAA and IBA (0.5, 1.5 and 3 mg/l) to determine the effect on root regeneration as well as the optimal concentration for various rooting techniques.

Observation recorded
Number of roots per shoot**,** Per cent (%) response.

Analysis of data
The experiments were conducted according to completely randomized design (CRD). Each experiment was replicated three times. The goal of the study was to find a significant difference between the treatment means. On the basis of critical difference, the treatment means were classified as significant or non-significant (CD).

3. Results and Discussion
3.1 Standardisation of callus induction and regeneration of banana *cv*. Grand Naine
Depending on the plants' nutritional requirements, various plants may have varied optimal development and tissue morphogenesis. Additionally, the requirements for optimal development may change for tissue from other plant sections. The original source of tissue culture media was nutrient solution used for growing whole plants. On callus formation media, the proportion of explants generating callus was significantly impacted by plant growth regulators (Saad and Elshahed 2012) [23].
In bananas, different types of explants have been used to induce callus and regenerate plants. Among the explants that have been used, immature male/female flower and *in vitro* shoot tip cultures are reported to be the most responsive for initiating regenerable, embryogenic cell suspension cultures

(Escalant *et al.*, 1994; Grapin *et al.*, 1998 Ganapathi *et al.*, 1999) [6, 9, 8]. Callus induction and regeneration of fertile banana plants plant develop in; i) crop improvement ii) mass propagation. It has been known that the potential for callus induction and regeneration in banana tissue culture depends on several factors, such as the genotype of the donar plant, the type and physiological status of the explant, the composition and concentration of the basal salts and the organic components and plant growth regulators in the culture medium.

Plant growth regulators play a vital role in plant tissue culture those are species specific. It is a critical media component in determining the developmental pathway of the plant cells/tissue. Ratio of auxins and cytokinins are the principal growth regulators in plants in the regulation of cell division and differentiation. They are usually used together for callus induction development. A high auxin to cytokinin ratio generally favours root formation, whereas ahigh cytokinin to auxin ratio favours shoot formation. An intermediate ratio favours callus induction and development. For induction of callus, different auxins such as IAA, NAA, 2,4-D, etc. and different cytokinin's like Kinetin, BAP etc. at varying concentrations were used. A low ratio is used for direct regeneration or induction of somatic embryos, from which whole plants can be subsequently produced.

3.2 Studies on standardization of callus induction from floral organ of banana *cv*. Grand Naine with different concentration of 2, 4-D

Immature male inflorescence buds were used as explant for callus induction. For the purpose of callus induction, the male inflorescence buds were dissected aseptically & inoculated on MS agar medium with 11 various doses of 2,4-D.The treatment CIM6 (MS + 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA) induced highest per cent of callus (85%) followed by CIM7 (MS + 3.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA) induced 66% callus. Since 2,4-D is utilised to induced and maintain callus formation in floral organ of banana male bud. Additionally, it stops premature regeneration from occurring, which can result in the loss of embryogenic competence. The cause may be related to 2,4-D's encouragement of tissues that are not organised (Strosse *et al.*, 2003) [26].

These results were in line with those of Azlin (2008) [1], who found that 2 mgL^{-1} 2,4-D-containing medium produced 53.9% of embryogenic callus. In MS medium supplemented with 2 mgL^{-1} 2, 4-D and 0.2 mgL^{-1} zeatin, Ganapathi *et al.* (2001) [7] got a 42% callus. In contrast, an SH-based medium supplemented with 2.0 mg/L of 2,4-D and 0.2 mg/L of zeatin successfully induced calluses at a rate of 39.5%. Meenakshi *et al.* (2007) [16] investigated concentration of auxin affected on development of calluses from immature male flowers of several banana cultivars that are members of the AAA genomic group. In 1.5 mgL^{-1} 2, 4-D, the cultivar Lalkela had the maximum callus induction (77.7%), followed by Shrimanti (52.2%), Basrai (51%) and Grand Naine (42.5%) in 2.5 mgL^{-1} 2,4-D. The lowest callus induction response was seen in 1.5 mgL^{-1} 2,4-D with the cultivars Ardhapuri (42%) and Mutheli (40%).

3.3 Days to callus induction

In the present investigation minimum number of days required for callus induction ranged from 13-24 days in explant. The minimum number of days (13) to callus induction recorded in treatment MS + 2.5 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA (CIM_6). Similar finding also reported by Prathibha *et al.*, (2014) [19] in Nanjangud Rasbale.

3.4 Weight of callus (mg) after 21 days of inoculation

The maximum of callus weight (2.1gm) was recorded in 21 days after inoculation in CIM_5 (MS + 2.0 mg l^{-1} 2,4-D + 0.5 mg l^{-1} BAP + 0.5 mg l^{-1} NAA) The cultured tissue grown on solid medium were carefully removed from the culture vessel and made free from specks of agar that had adhered at the point of contact. Accumulated water was blotted out gently without squeezing the tissue. The tissue was then transferred onto pre weighed aluminum foil and the weight was determined on a single pan digital balance. The weight of callus produced ranged from 0.33g to 2.1g. Better results of callus weight was recorded in shoot explant i.e., 2.1g on media combination of Similar results also reported by Meenakshi *et al.* (2010) [15].

Fig 3: Callus induction

3.5 Regeneration through callus

3.5.1 Shooting

A wide range of physical and chemical variables combine steadily and synergistically to form *in vitro* organogenesis, a hormonal change in explant & transition. The shift in the tissue's architectural pattern caused by cell division reflects the explant's transition into a proliferating callus mass under cultural conditions. The plant genotype, explant origin and physiological condition of the cultured tissue these all factors that affect callus induction (Murashige, 1974) [30]. Plant hormones are thought to be the primary factor in the explants' establishment, stimulation of numerous shoots and eventual *in vitro* plantlet development. Particularly, it has been discovered that cytokinin may promote auxiliary shoots from meristematic explants (Madhulatha *et al*. 2004) [13]. Our investigation medium containing MS + 4.5 mg l^{-1} BAP proved to be the best for regeneration of shoot within 22 days.

Required for shoot proliferation. (79.34%) Maximum shoots was recorded in same medium (16.75).

Present results are partly consistent with those of Ali *et al.* (2011) [2] who claimed that the optimum multiplication response for shoot multiplication observed in by MS medium with 1.0 mg/l BAP+ 0.25 mg/l Kinetin. The regeneration and multiplication of shoots were strongly impacted by various concentrations of BAP. Several workers reported similar outcomes for regeneration of shoots (Kalimuthu *et al.* 2007) [12]. However, in MS medium increasing concentration of BAP decrease shoot multiplication and shorter shoot length. According to Arinaitwe *et al.* (2000) [3], banana cultivars responded to BAP more significantly than other cytokinins (ZN, KIN & 2-iP).

Fig 4: Regeneration through callus

3.5.2 Rooting

Auxin play important role for induction & development of roots. A high auxin to cytokinin ratio promotes root regeneration. However, auxins alone themselves can cause the tissue cultured branches to produce roots. Auxins have been discovered to have an impact on cell growth, root initiation and the suppression of lateral buds (Cronauer and Kirikorian, 1984, Jarret *et al.* 1985) [5, 11].

MS + 0.5 mgl^{-1} NAA + 1.5 mgl^{-1} IBA + 1g/l activated charcoal applied in MS medium in per form best than other treatment. Highest percent rooting (75.93), root frequency, number of roots per shoot (14.8) and root length (6.1). Were observed in same medium. Also, our finding was consistent with (Arinaitwe *et al.* 2000) [3].

Our findings are likewise supported by Gubbuk and Pekmezci (2006) [10] and Roy *et al.* (2010) [22], who achieved rooting using 1.0 mg/l IBA and 200 mg/l activated charcoal.

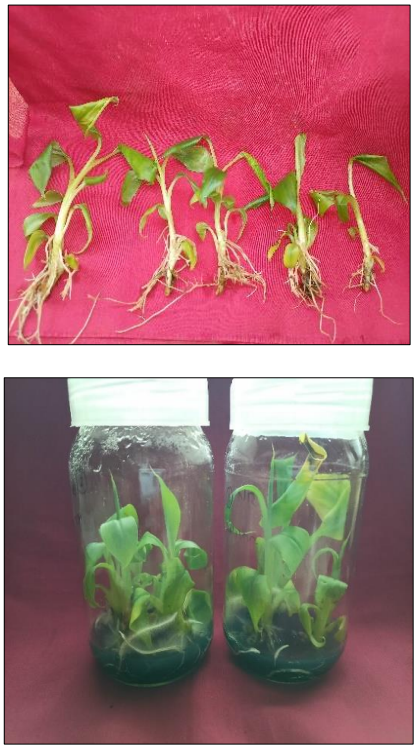

Fig 5: Induction & development of roots

3.6 Hardening

Hardening of *in vitro* raised plantlets with minimum mortality. The *in vitro* derived plantlets must acclimate to the altered environment present *in vivo* since they are ill-equipped to withstand the low relative humidity, increased light intensity and greater temperature, among other factors.

The rooted plantlets were placed in terrariums with various potting mixture. The survival of plantlets is improved by covering them with polythene, which raises the relative humidity. The highest plant survival percentage (80%) was recorded in HPM_3 (88.89). Other potting combinations have comparatively poor survival rates. The deterioration at the collar area and subsequent withering of the shoots were the causes of the mortality shown here. Our findings also corroborated the findings of several other studies who came to the same conclusion that gradual acclimation of tissue culture plants from high humid conditions in the pots is a crucial phase for their survival in many fruit crops, including banana (Saindane *et al.*, 2001, Oliveira *et al.*, 2001, Youmbi *et al.*, 2005 and Vasane and Kothari, 2006) [24, 17, 28, 27]. The resulting plantlets were hardened and successfully planted in the field.

4. Conclusion

In conclusion the outcome of the present study indicates that, endogenous level & exogenous supplied plant growth regulator play important role for regeneration of plantlets.

Finding of the present study elucidate that plant regeneration could be possible from calli developed from immature male flowers of banana. Although the frequency of callus induction and plant regeneration from immature male flowers in the present study are not so high, there is still a scope to improve organogenesis by further manipulation of the medium composition and culture conditions which used be suitable for improvement in G-9 Cultivar.

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