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In vitro Callus Induction of Green Bamboo

A. S. Mali*, N. S. Chavan and N. R. Chavan

MGM College of Agricultural Biotechnology (CABT), Vasantrao Naik Marathwada Krishi Vidyapeeth, Gandheli, Aurangabad-431003, Maharashtra, India

**Corresponding author*

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A B S T R A C T

Bamboo farming is one of the most promising business in India due to high productivity and assured yield under adverse conditions. The increasing demand of bamboo plantlets can be not be met by traditional cultivation methods. Plant tissue culture techniques have potential for producing large number of healthy plantlets for ever-growing demand. Hence for *in vitro* culture techniques induction of callus is necessary which can be further used for organogenesis and somatic embryogenesis. The present study demonstrates a simple and efficient method for callus induction of Bamboo. The leaf, node and seed are used as explants for induction of callus. These explants were subjected to sterilization treatment and it is found that Tween -20, bavistin and ethanol are sufficient for proper sterilization of explants. Then these explants were subjected to various concentrations of growth hormone 2,4-Dichlorophenoxyacetic acid (2,4-D) added in Murashige and Skoog Media. It was concluded that the seeds are suitable explants for rapid induction of callus. Then the various concentration of 0 to 8 mg/lit of 2,4-Dichlorophenoxyacetic acid was used and it was observed that 5 mg/lit concentration is best suited for growth of callus under in vitro condition when all other parameters are kept constant.

Introduction

Bamboo is a fast-growing plant. Bamboo plants distribute from tropical to temperate zones of Southeast Asia and they provide useful resource for local economies (Chang *et al.,* 1991 and Chang *et al.,* 1997). Bamboo firstly originated in China over 5000 years ago. Han Dynasty (206 to 221 BC) starts the use of bamboo to make paper and books. There are 1000 species and 91 genera of bamboo. *Dendrocalamus strictus, Bambusa arundinacea* and *Bambusa vulgaris* are among the important species found in India. In bamboo genetic resource India is second largest after China. India's natural resources of bamboo, consisting of a large number of species, constituents one of the world's largest reserves of this commodity. In India out of total forest area of 75 million hectares bamboos, natural and planted, occupy 10 million hectares. About 9.5 million tones is the annual production of bamboo in India (Varmah *et al.,* 1981).

The most important use of bamboo is as a raw material in pulp, about 1.9 million tones used by pulp industries. About 4.9 million metric

tonnes are being presently utilized for paper making (Varmah *et al.,* 1981). In the lives of rural poor and rural industries the bamboos occupy a special place, especially in Asia (Rao *et al.,* 1990). Young new shoots of bamboo are eaten in Asia. In India bamboo is used for making paper, since it provides good quality paper pulp (Mehra *et al.,* 2007). Bamboo can be used as materials for house construction, daily sundry goods, agricultural and fisheries tools, and crafting material (Chang *et al.,* 1997).

It has traditionally been propagated through seed or through vegetative methods but these methods besets with many problems. Conventional methods of propagation are best with problems such as seed sterility, non-availability of seeds, unpredictable and long flowering cycles and bulkiness of rhizomes (Rao *et al.,* 1990). Rhizome planting, culm cutting and seedling cultivations these are the methods for the multiplication of bamboos (Kumar *et al.,* 1981 and Uchimura, 1990). Therefore, it is imperative by adopting the latest plant biotechnologies, new utilities of bamboos could progress beyond the current level. Tissue culture is one essential technique to micro propagate regenerated plant tissues and it is also for the genetic improvement through the use of different transformation strategies (Kalia *et al.,* 2004; Lin *et al.,* 2003, 2004; Sexena 1990). In this regard, tissue culture techniques offer an alternative method for rapid multiplication of bamboo species and tissue culture extremely useful (Kondas 1982).

The callus culture systems useful for plant improvement, however, it will depend on the ability to regenerate whole plants from callus. In general bamboo has been considered difficult to culture *in vitro* (Alexander *et al.,* 968) reported aseptic culture of embryos from zygotic embryo culture, while callus cultures derived from the seed, leaves and shoot tips of several species were also produced (Huang and Murashige1983). Regeneration of callus induction from mature seeds was observed in some species of bamboo (Rao *et al.,* 1985; Yeh and Chang 1987).

The potential of micro propagation has raised high hopes and a lot of research has been focused on the development of protocols for rapid and large scale propagation (Rao *et al.,* 1985; Nadgauda *et al.,* 1990; Godbole *et al.,* 2002; Sood *et al.,* 2002). Micro propagation of bamboo species using seed has the advantage of having greater number of genotypes in culture, from where propagation may proceed ensuring greater diversity of the species.

However, micro propagation through auxiliary branching is expensive, and hence not applicable for plant production for forestry and silviculture, especially in rural regions in Asia. Therefore it is desirable to develop methods based on somatic embryogenesis to reduce costs of bamboo liners and planting materials for forestry. Somatic embryogenesis is a promising alternative wherein somatic embryos can be germinated to form plants (Rao *et al.,* 1987., John *et al.,* 1995., Joshi *et al.,* 1997). The first report on SE in bamboo was published almost 25 years ago (Mehta *et al.,* 1982), and since then more than 50 publications have dealt with SE in bamboos.

Thus, somatic embryogenesis can be effectively used for mass propagation of important bamboos. Since, embryogenic calli maintain their regenerative competence for a long time, they can give rise to genetically uniform plantlets (Vasil *et al.,* 1982). The compact organized structures can be visibly screened, multiplied and germinated easily and these add to list of benefits of using somatic embryos. SE serving as a preferred method for rapid *in vitro* multiplication of

plants, it also serves as are ideal experimental system for understanding mechanism of differentiation as well as totipotency in plant cells (Arya *et al.*, 2000). It is also serving as ideal explants for carrying out transformation studies and regeneration of transgenic plants.

However, in almost all cases the research has been limited to SE from seeds or seedlings in two species, namely *Dendrocalamus strictus* and *Bambusa bambos*, for which seeds are available annually.

In India, *Dendrocalamus strictus* is the most economically important bamboo species, accounting for 66.6% of the raw material used for paper production material. Induction of callus is used for indirect somatic embryogenesis. By considering above points the present investigation entitled "*In vitro* callus induction of green Bamboo" was carried out at MGM College of Agricultural Biotechnology, Gandheli, Aurangabad.

Materials and Methods

Experimental site

The research work was carried out in MGM College of Agricultural Biotechnology, Gandheli, Aurangabad.

Experimental Design

The experimental was laid down in Completely Randomized Design (CRD) With nine treatments of 2,4-D ranging from 0 mg/lit to 8 mg/lit and three replication.

Plant Material

The most important material to initiate callus is suitable explants. These explants should be obtained from mother plants which are healthy, disease free and age of mother plant should be at least 2 years. Healthy Explants were obtained from the field of Mahatma Gandhi Missions Hills, Gandheli, Aurangabad. However the seeds of green bamboo were purchased from nursery. The mature seed explants of *Dendrocalamus strictus* variety of bamboo purchased from RK nursery and seeds, 7th street, Shree Ram Nagar, Vadavali Coimbator-641 046.

Sterilization of explant

The explants sterilization is of at most important to avoid the growth of unwanted microorganism under *in vitro* condition. These explants were collected from healthy mother plants. Then these explants are washed with RO water to remove dirt. Then the explants were washed with sterile distilled water for three times. After this the explants were transferred in Laminar Air Flow Cabinet for further sterilization. Inside Laminar Air Flow Cabinet these explants were again washed with bavistin for 10 minutes followed by tween -20 for 5 minutes followed by three washes of autoclaved distilled water. After the explants were treated with 70% ethanol for 30 seconds and then rinsed with sterile distilled water for three times. These explants are suitable for inoculation. (Yuan *et.al.* 2009)

Media Preparation

The Murashige and Skoog basal media was prepared by adding various components from stock solution in autoclaved distilled water. The concentration of 2,4- Dichlorophenoxy acetic acid was ranged from 0 to 8 mg/l. These stock of 10 mg/lit of 2,4-D was prepared by dissolving powder in 1 N sodium hydroxide . The concentration of Sucrose was 30 gm/ lit and clarigel at concentration of 3g/lit was used for solidification. The pH of the medium was adjusted to 5.8 before addition of agar. The 40 ml media was poured into each culture bottles. They were then autoclaved at 121℃ for 20 minutes at 15 psi

pressure and transferred to culture room where they were kept under dark for 72 hours before inoculation (Murashige and Skoog 1962) (Gillis *et al.*, 2007).

Inoculation of explant callus induction

Leaf:- The surface sterilized explants of leaf was cut to size of 1 cm^2 under Laminar Air Flow Cabinet. Then these explants were damaged by making cuts. Then these explants were transferred on surface of MS media.

Node: The sterilized nodes were further trimmed to reduce its size and nodal section is damaged using blades under LAF cabinet. These explants was transferred to MS media and submerged in it.

Seed: The surface sterilized seeds explants contain seed covering which was removed using forceps. These removals were carried out under LAF cabinet. After removal of seed covering the seeds were transferred to MS media for further incubation. (Lin *et.al.* 2004)

Culture condition for callus induction

After inoculation of explants these culture bottles were capped and sealed with parafilm strips. These culture bottles were transferred to culture room. All the cultures were incubated in dark condition in a culture room at 25 ± 2ºC, with a relative humidity of 50-60 percent.

Observations and Analysis

The observation includes number of days required for germination of seeds, Days required for initiation of callus, diameter of callus, nature of callus and colour of callus. The quantitative data obtained on various observations was analyzed by "Analysis of variance (ANOVA)" method (Panse and Sukhatme, 1967).

Results and Discussion

Surface sterilization

The surface sterilization of explants is very essential for successful establishment of plant tissue culture. The protocol for surface sterilization was successfully established. Explants are washed with RO water to remove dirt. Then the explants were washed with sterile distilled water for three times. After this the explants were transferred in Laminar Air Flow Cabinet for further sterilization. Inside Laminar Air Flow Cabinet these explants were again washed with bavistin for 10 minutes followed by tween -20 for 5 minutes followed by three washed of autoclaved distilled water. After the explants were treated with 70% ethanol for 30 seconds and then rinsed with sterile distilled water for three times. It was found that the explants reminded sterile under *in vitro* condition without any sign of contamination for 21 days.

Suitable explants for callus induction

Total three different explants were used to initiate the callus culture *Viz.* Leaf, Node and Seed. It was found that both leaf and node fail to produce the callus under given condition and callus formation was observed from seeds after germination. The reason that leaf and nodal explants fail to form callus is lack of regeneration under provided culture condition. The leaf and nodal explants survived culture condition only up to seven days. After seven days most of them died due to browning. The explants fail to endure sterilization treatment.

Days required for germination

Data of average number of days required for germination of seeds as influenced by different concentration of growth hormone

2,4-Dichlorophenoxyacetic acid is given in table 1.

Data presented in table 2 and depicted in Fig. 1 revealed that the germination of bamboo seeds was influenced by different concentration of 2,4-D. The treatment T_4 is significantly superior over T_1, T_2, T_3, T_6, T_7, T_8 and T_9 . Treatment T_4 is at par with T_5. It was observed that Treatment T_5 is at par with treatment T_6, T_3 and at par with rest of others. Moreover Treatment T_6 is superior over T_8, T_9, T_1 and T_2. The concentration of 2,4-D (3mg/lit) is most suitable for germination of seeds. But it is also observed that there is breaking of seed dormancy at this concentration and after few days if these are not transferred to light, the shoot fails to grow and forms callus. Hence *in vitro* germination of seed provide a more aseptic way of forming callus as seed has hard seed coat which can endure harsh sterilization treatment which generally is not tolerated by leaf and nodal explants. The seeds are very attractive source of initiation of callus culture for bamboo.

Table.1 Different concentrations of 2,4-Dichlorophenoxyacetic acid

Treatments	Concentrations of 2,4-D (mg/lit)
T_0	0
T_1	1.0
T_2	2.0
T_3	3.0
T_4	4.0
T_5	5.0
T_6	6.0
T_7	7.0
T_8	8.0

Table.2 Average number of days required for germination of seeds

Treatment (Concentration of 2,4-D)	Average Number of Days required for germination of Seeds
T_1(0.0mg/lit)	8.67
T_2(1.0mg/lit)	8.33
T_3(2.0mg/lit)	5.67
T_4(3.0mg/lit)	3.00
T_5(4.0mg/lit)	3.33
T_6(5.0mg/lit)	4.67
T_7(6.0mg/lit)	5.67
T_8(7.0mg/lit)	6.00
T_9(8.0mg/lit)	6.33
C.D. (0.01)	**1.430**

Table.3 Average number of days required for initiation of callus

Treatment (Concentration of 2,4-D)	Average Number of Days required initiation of callus
T_1(0.0mg/lit)	Fail to initiate callus
T_2(1.0mg/lit)	17.33
T_3(2.0mg/lit)	17.33
T_4(3.0mg/lit)	18.00
T_5(4.0mg/lit)	18.33
T_6(5.0mg/lit)	22.33
T_7(6.0mg/lit)	24.33
T_8(7.0mg/lit)	31.00
T_9(8.0mg/lit)	30.67
C.D. (0.01)	**1.885**

Table.4 Diameter of callus

Treatment (Concentration of 2,4-D)	Diameter of callus (mm)
T_1(0.0mg/lit)	Fail to initiate callus
T_2(1.0mg/lit)	1.00
T_3(2.0mg/lit)	3.00
T_4(3.0mg/lit)	1.67
T_5(4.0mg/lit)	4.00
T_6(5.0mg/lit)	1.67
T_7(6.0mg/lit)	1.67
T_8(7.0mg/lit)	1.33
T_9(8.0mg/lit)	1.33
C.D. (0.01)	**1.089**

Fig.1 Effect of different concentration of 2,4-D auxin on seed germination

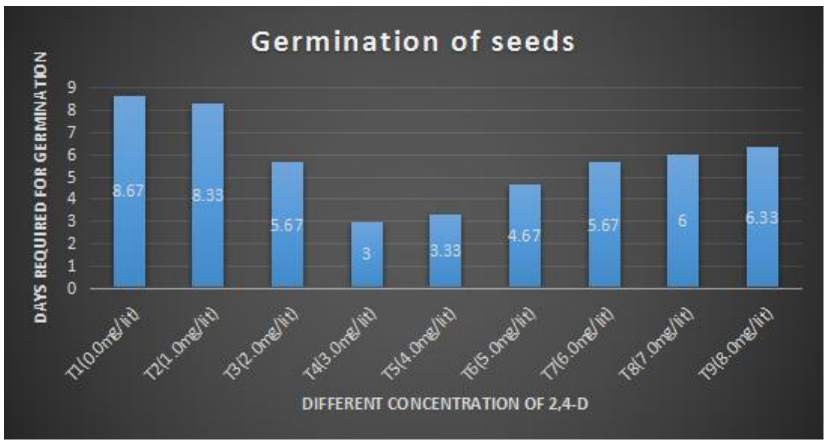


Fig.2 Number of days required to initiate callus

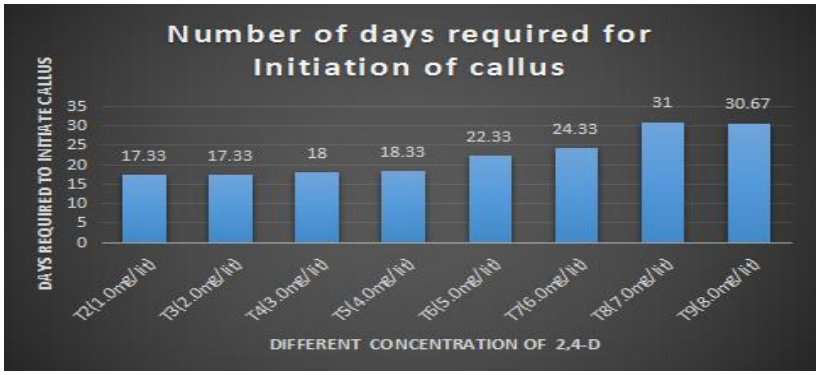


Fig.3 Effect of different concentration of 2,4-D on callus growth

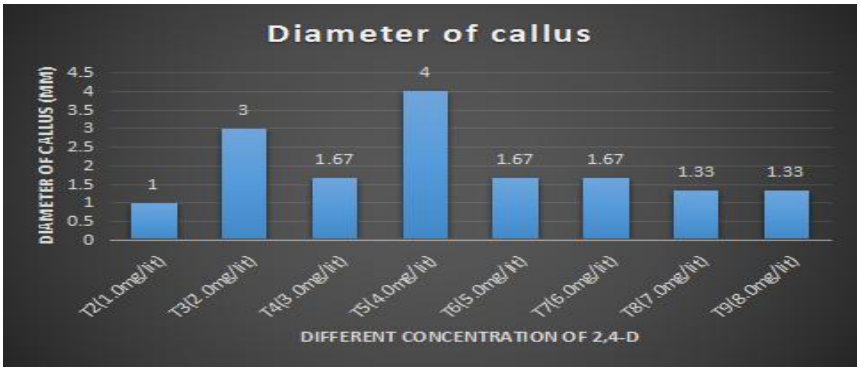
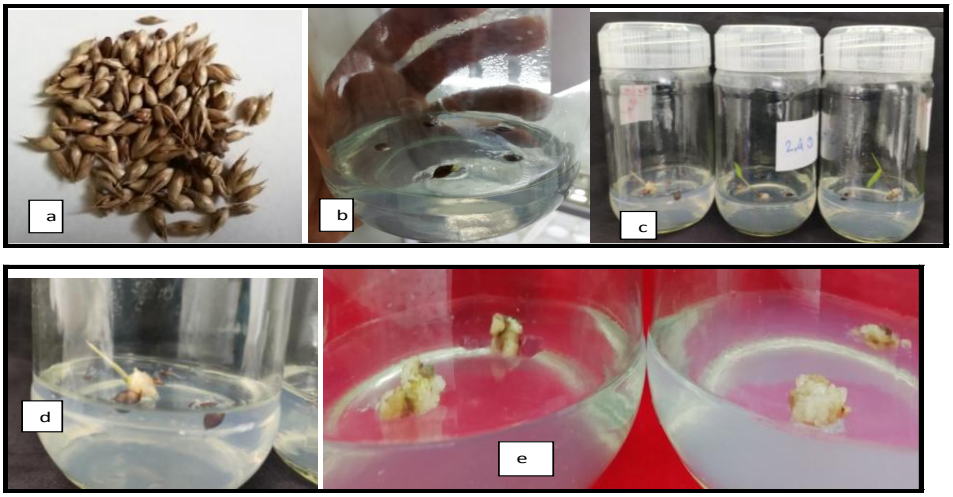


Plate 5 Growth of callus



a = **Seed explants of Bamboo (Dendrocalamus strictus) .b=noculation of seed .c=Germination of seed explants . d= callus initiations . e = The friable, light yellow whitish callus**

Days required initiation of callus

Data of average number of days required for initiation of callus as influenced by different concentration of growth hormone 2,4-Dichlorophenoxyacetic acid is given in table 3.

Data presented in table 3 and figure 2 clearly indicates that the initiation of callus from germinated seed is not observed in treatment T_1. This indicates that growth hormone is necessary for formation of callus as other treatment successfully initiated callus. The 2,4-dicholorphenoxy acetic acid induce dedifferentiation in cells of explants which form unorganized mass of cells. This unorganized mass grows with each day till supply of sufficient nutrient from media. Treatment T_2, T_3, T_4 and T_5 are at par with each other for initiating callus where as they are superior over rest other treatments. This experiment indicates that concentration of 2,4-D at 1mg/lit, 2mg/lit, 3 mg/lit is most suitable for inducing callus from seed explant. Whereas other concentrations do induce callusing but they need more time. Callus induction is result of threshold concentration of this growth hormone inside and outside the explant under *in vitro* condition

Diameter of callus

Data of average diameter of callus as influenced by different concentration of growth hormone 2,4-Dichlorophenoxyacetic acid is given in table 4.

Data presented in table 4 and figure 3 shows that the growth of callus is significantly influenced by different concentrations of growth hormone 2,4-D. The diameter of callus is found highest 4mm in treatment T_5 which is significantly superior over rest of others. Moreover the treatments T_3 found significantly superior over rest of others except T_5. All other treatments are at par with each other. It has been observed that there is not specific pattern of callus growth. The growth of callus is due to threshold level of hormones present inside media as well as synthesized by callus itself.

Nature of callus

The callus produced under *in vitro* condition is friable and easily breaks. There is no effect of growth hormone concentration of nature of callus. This friable callus can be used for suspension culture. Moreover it can be further used for somatic embryogenesis.

Colour of callus

The callus when initiated forms completely whitish mass but as it growth continues it turns slightly yellowish. The colour of callus is found to be same in all treatment. Hence it is concluded that the different concentration of growth hormone does not affect colour pattern of callus.

The result obtained in this research work are clearly indicates that 2,4-dichlorophenoxy acetic acid is suitable for induction and growth of callus of bamboo. Similar type of result was obtained by Yuan *et al.*, (2009) in *Bambusa multiplex.* This callus can be further used for induction of Somatic embryogenesis. Moreover Yeh *et al.*, (1984) also regenerated callus using bamboo explant and utilized it for plant regeneration via somatic embryogenesis. The calli formed is fragile and it represents that the cells are actively growing. The induction of callus formation was also observed under influence of 2,4-Dicholophenoxy acetic acid. This hormone increases the rate of cell division in explant. This starts the process of dedifferentiation where unorganized mass of cells start forming under *in vitro* condition from differentiated explant. This process once started leads to

formation of calli. Many more researchers' *viz*. Zhang *et al.,* (2010), Rao *et al.,* (1985), Godbole *et al.,* (2002) etc. Reported callusing induction in different species of bamboo. The produced calli shows whitish mass of cells. These calli when matures leads to yellowish colouration. The growth of callus again starts under influence of growth hormone The fundamental work carried out in this research paper clearly indicated that seed are best source to obtain callus of bamboo which can be further used for somatic embryogenesis.

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