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In Vitro ESTABLISHMENT OF SHOOT CULTURE FROM AXILLARY BUD OF *Dendrocalamus strictus* AND MULTIPLICATION OF ITS SHOOT

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ABSTRACT

***Dendrocalamus strictus*, also known as solid Bamboo found in the natural vegetation of Tropical, Subtropical, and wild Temperature regions. It is one of the Bamboo species of the National Bamboo Mission of India. It is a great source of the economy as it contains 85% cellulose, which is used for the Paper and Pulp Industry. Bamboo is the so-called poor man's Timber as it is the backbone of the circular economy of South-West Asian countries. This study has the main objective to develop a reliable Protocol for the establishment of Shoot culture from Axillary Bud of *Dendrocalamus strictus* and *in vitro* multiplication of its shoot. This *in vitro* Propagation has emerged as a Promising technique for Mass Propagation of elite Bamboos in a stipulated period and can lead to the production of Healthy, Disease-free Plants and the multiplication can occur throughout the year irrespective of the Season.**

KEYWORDS: *Dendrocalamus strictus,* Organogenesis, Multiplication of Shoots

Bamboos represent one of the world's most important natural and renewable bioresources and are considered among the most useful plants known to mankind. Bamboos are short rotation, arborescent, monocotyledonous, perennial flowering plants in sub-family Bambusoideae of the grass family Poaceae. The somatic chromosome number of various bamboo species varies between 12-72, but most of them are polyploids and natural populations are highly heterogeneous. They contribute a major part of the natural vegetation of tropical, sub-tropical, and wild temperate regions. There are about 88 genera and 1575 bamboo species distributed worldwide covering an area of more than 14 million hectares and area under bamboo confined to South and South-east Asia (Wu and Raven, 2006). Herbaceous bamboos with approximately 110 species were mostly found in Brazil, Mexico, Argentina, and West Indies (Judziewicz *et al.*, 1999). Asian countries such as Nepal, Laos, Vietnam, Bhutan, Thailand, India, China, Bangladesh, and Myanmar account for about 1000 species. Out of which China has the richest bamboo resource having 34 genera and 539 species and large bamboo industry worldwide having a total of about 5 million hectares of bamboo forests (Maoyi 1998 and Bystriakova *et al.*, 2003). India (Jammu and Kashmir, Sikkim, Himachal Pradesh, Tripura, Mizoram, Assam, Nagaland) is second after China in bamboo resources having 125 species belonging to 23 genera and produce about 4 to 6 million tonnes of bamboos annually out of which 2.2 million tonnes used in the paper industry (Sharma, 1980 and Gillis *et al.,* 2007). In Himachal Pradesh, 8 species of bamboo have been reported under 5 genera in Nalagarh, Nurpur, Nahan, Palampur, Kunihar, Dehradun, and Paonta which include important edible species *Bambusa nutans*, *Dendrocalamus hamiltonii*, *Dendrocalamus parishii*, *Dendrocalamus strictus*, *Phyllostachys aurea.*

Dendrocalamus strictus also known as solid bamboo, Male bamboo or Culcutta bamboo is a tropical and subtropical clumping species native to southeast Asia. *Dendrocalamus strictus* is accredited as one of the priority bamboo species by National Bamboo Mission. The species generally grows on hill slopes, sea level up to 1,200m. It is native to Asia including the Indian subcontinent and Indo-China (Clayton *et al.,* 2015). The demand for *Dendrocalamus strictus* is increasing day by day as it contains 85% cellulose and is used for the paper and pulp industry (Kapruwan *et al.*, 2014). Bamboos form the backbone of the rural economy of South-East Asian countries, sustaining the lives of millions of people and often called "poor man's timber" (Yeasmin *et al.,* 2014).

Dendrocalamus strictus is a medium-sized bamboo with culms of about 8-20m tall and 2.5-8cm in diameter. The internodes are 30-45cm long and are thick-walled. This species has pale blue-green culms when young, and dull green or yellow culms on maturity, which appears in a zig-zag manner from the middle towards the top. Branching occurs from the base to the midculm.

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Leaves are linear-landscaped and size is variable as they are bigger in moist areas and smaller in dry areas, size may vary between 5-25cm long and 1-3cm broad, rounded at the base into a short petiole, the tip is sharply tapering with a twisted point. The gregarious flowering size of male bamboo varies from 25-45years. Sporadic flowering is seen almost every year. Flowers appear from November to February and fruits are seen from February to April.

Bamboo is an extremely diverse plant, which easily attains different climatic as well as soil conditions. People from different regions address bamboos in different names because of their multipurpose properties such as Chinese called bamboo as "Friends of the people", Vietnamese as "My brother" and Indians as "Green gold". Young shoots are regarded as one of the most useful health foods as the shoots are rich in various amounts of protein, vitamins, carbohydrates, fibers, minerals, and very low fat. Bamboo shoots are properly boiled before consuming because the freshly harvested shoots may contain high content of toxic cynogenic glycosides (taxiphyllin) which may lead to serious health threats. The presence of phytosterols and high fiber in bamboo shoots reduces fat and cholesterol levels of blood and protects our body from coronary diseases and potential carcinogens, presence of tyrosine facilitates biochemical metabolism of the body. Tabisheer (bamboo manna or bamboo silica) is one of the main substances from bamboo used in Ayurvedic and Tibetan medicine. It is considered an anti-inflammatory, antispasmodic, febrifuge, astringent, and tonic for the lungs. Sitopaladi churna (made from tabisheer) is used traditionally for the treatment of tuberculosis and has been adopted as a popular remedy for sore throat, sinus congestion, common cold, bronchitis, and asthma. Additionally, new products such as bamboo vinegar, bamboo charcoal, bamboo juice have been developed. Bamboo vinegar is used as a traditional medicine for stomach-related infections. Modern manufacturing techniques also allow the use of bamboo in timber-based industries to provide bamboo flooring, house constructions, board products, handicrafts, agriculture, fisheries tools, laminates, and other household items (McNeely, 1995). *Dendrocalamus strictus* is also considered as the drought-tolerant bamboo so it is extremely suitable for areas with low rainfall. Bamboo is becoming a substitute for wood in pulp and manufacturing, about 25% of the fiber used in the Indian paper industry each year comes from bamboo (Anon, 1998). Bamboo is an efficient agent for preventing soil erosion and conserving soil moisture (Christanty *et al.,* 1996).

The plant tissue culture technique offers an advantage over traditional methods. Micropropagation of bamboos through tissue culture provides an excellent opportunity as an alternative biotechnological tool having high multiplication rates to produce a large number of plants of improved quality and shortened rotation cycles. *In vitro* propagation has emerged as a promising technique for mass propagation of elite bamboos in a stipulated period and can lead to the production of healthy, disease-free plants, and the multiplication can occur throughout the year irrespective of the season. Stocks of germplasm can be maintained for many years in form of *in vitro* cultures and it also facilitates the international exchange of germplasm without the inherent risk of spreading pests (Sun *et al.,* 2008).

At present Government of India is running National Bamboo Mission to support and encourage bamboo cultivation in India. The main objective of this mission is to increase the productivity of commercially important bamboo species and shoots within the stipulated time for regular supply to the processing units for the production of more value-added products. National Bamboo Mission (NBM), Department of Biotechnology (DBT), and Indian Government promoting cultivation and improvement of 15 industrially important species in the country (Swarup and Gambhir, 2008).

List of Abbreviations

Abbreviation	Full form
MS	Murashige and Skoog medium
v/v	Volume by Volume
IAA	Indole-3-acetic acid
BAP	Benzyl amino purine
BA	6-Benzyl adenine
2,4-D	2,4-Dichlorophenoxyacetic acid
IBA	Indole-3-butyric acid
°C	Degree celcius
et al	Co-workers
$HgCl_2$	Mercuric chloride
Kn	Kinetin
NAA	Napthalene acetic acid
mg/l	Milligram per litre
TDZ	Thidiazuron
µM	Micro molar

MATERIALS AND METHODS

Source of Plant Material

Young and healthy plants of Dendrocalamus strictus were procured from CSIR-Institute of Himalayan Bioresource Technology, Palampur (H.P.). Axillary buds are used as the explants for the present research, which has been taken from the young field-grown plants of *Dendrocalamus strictus.*

Plant Tissue Culture Laboratory

The present research on bamboo species *Dendrocalamus strictus* was carried out in the Department of Biotechnology, Shoolini Institute of Life Sciences and Business Management, Solan (H.P.).

The methodology used for various objectives has been described under the following headings:

Sterilization and Cleaning of Glassware and Equipment

The glasswares (test tubes, beakers, Petri plates, flasks, glass pipettes, etc.) and equipment (Scalpel, forceps, Surgical blades, etc.) were taken and washed properly with laboratory reagents with the help of a cleaning brush. The glasswares were rinsed with double distilled water and sterilized in a hot air oven for about 1-2 hours at 160ºC. The test tubes which got contaminated by the microorganisms were first steam-sterilized in an autoclave to kill microorganisms and then washed with detergents, followed by dry heat sterilization in the oven. The working area of laminar airflow was firstly surface sterilized with 70% ethanol and UV radiations.

Composition of Nutrient Medium

The culture medium used for in vitro growth of the plant is composed of several components such as the complex mixture of various salts (essential elements or mineral ions), organic supplements (vitamins and amino acids), carbon source (sucrose), gelling agents, plant growth regulators, distilled water, etc. For the proliferation of in vitro shoots a standardized MS medium was used and supplemented with various concentrations of growth regulators varying from 0.1-20mg/l. The composition of MS medium was as follows in table 1.

Preparation of Stock Solutions

The stock solution is a solution, which is diluted to some lower concentration for actual use. The stock solutions were prepared by dissolving each component of standardized MS medium separately and then mixing with other components and named it as a stock solution (A, B, C, and D). Separate stock solutions were prepared for each growth regulator by dissolving it in a minimal quantity of appropriate solvent (if insoluble in water) and then the final volume was made with distilled water. Concentrated stock solutions of analytical grade chemicals were stored in proper glass bottles in the refrigeration unit at 4ºC temperature. Before using the stocks for the preparation of MS medium, the bottles were shaken gently and if precipitation occurs they were immediately discarded. The advantage of stock solutions is that a small amount can be withdrawn whenever required for the preparation of medium.

Table 1: Composition of MS Medium

Inorganic constituents	Quantity(mg/l)
Major constituents	
NH_4NO_3	1650
KNO_3	1900
$CaCl_2.2H_2O$	440
$MgSO_4.7H_2O$	370
KH_2PO_4	170
Minor constituents	
H_3BO_3	6.2
$MnSO_4.4H_2O$	22.3
$ZnSO_4.7H_2O$	8.6
KI	0.83
$Na_2MoO_4.2H_2O$	0.25
$Na_2EDTA.2H_2O$	37.3
$FeSO_4.7H_2O$	27.8
$CoCl_2.6H_2O$	0.025
$CuSO_4.5H_2O$	0.025
Organic constituents	
Pyridoxine HCL	0.5
Glycine	2.0
Thiamine HCL	0.5
Nicotinic acid	0.5

Preparation of Medium

For the preparation of medium, the appropriate quantities of various stock solutions i.e. A, B, C, and D including growth regulators were added in a beaker. The final volume of the medium was adjusted with distilled water and sucrose as per the requirement of the experiment is added into it. After mixing the pH of the medium was adjusted to 5.6-5.8 with the help of 1N NaOH and 1N HCl. Required quantity of agar-agar was then added and dissolved by heating in microwave oven until complete dissolution takes place. Then the hot medium was poured 15-25ml in culture test tubes with constant shaking and the mouth of each test tube was closed with cotton plugs wrapped with the muslin cloth. The culture tubes containing medium were sterilized by

autoclaving at 121ºC for 15-20 minutes. The medium was allowed to cool at room temperature.

Aseptic Manipulation and Culture Conditions

To perform any practices and procedures in plant tissue culture to prevent the rate of contamination is referred to as aseptic conditions. Plant tissue culture media, which is rich in sucrose and other organic nutrients, supports the growth of many microorganisms (bacteria and fungi). It is, therefore, essential to maintain a completely aseptic environment. All the operations were carried out aseptically in the laminar airflow cabinet. Before culturing the surface of laminar airflow was properly wiped out with 70% ethanol. After that the medium and culture equipment was kept inside the laminar airflow cabinet and UV light was kept on for 15-20minutes.

After switching off the UV light, laminar airflow was allowed to use for further procedure. The instruments used for aseptic manipulation such as scalpels, forceps, surgical blades, etc were sterilized before each inoculation by dipping it into ethanol, followed by flaming and cooling, to avoid the chances of any contamination. The optimized conditions were applied for initiation of shoot culture are light and temperature conditions. After inoculation, all the cultures were incubated in a growth room at an optimum temperature of 25±2ºC and a uniform photoperiod of 16 hours of light and 8 hours of darkness was maintained for experimental cultures.

Surface Sterilization of Explants

The surfaces of plant parts carry a wide range of microbial contaminants, to avoid this source of infection, the plant tissue must be thoroughly surface sterilized before culturing it in the nutrient medium. The collected explants used for culturing were surface sterilized by various sterilizing agents. The explants used were washed in running tap water for about 15-20 minutes, followed by treatment with laboratory detergent tween-20 and aqueous solution 0.5% of Bavistin. Subsequently, explants were treated with 0.1% mercuric chloride and 70% ethanol inside the laminar airflow cabinet then the surface-sterilized explants were rinsed 3-4 times with distilled water and inoculated on the culture medium.

Initiation of Shoots in the Nutrient Medium

There are two different ways for the formation of shoots i.e. direct and indirect organogenesis. In this experiment, direct organogenesis was found to be the best method, from which the shoots have been induced. The explants which were surface sterilized by various chemical treatments were inoculated in MS medium containing various combinations of BAP, NAA, and KIN for the initiation of shoots. These growth regulators are used at different concentrations which in turn enhance the shoot initiation (Table 2). The inoculated explants were kept in a culture growth room and favorable culture conditions of temperature and photoperiod were maintained for the initiation of shoots.

Table 2: Effect of different concentrations and combinations of growth regulators on shoot initiation of *Dendrocalamus strictus*

Media	The concentration of Growth Regulators (mg/l)		
	BAP	NAA	KIN
MS_1	0.5	0.2	-
MS_2	1.0	0.2	-
MS_3	1.5	0.2	-
MS_4	2.0	0.2	-
MS_5	2.5	-	0.5
MS_6	3.0	-	0.5
MS_7	3.5	-	0.5
MS_8	4.0	-	0.5

In vitro* multiplication of *Dendrocalamus strictus

Different concentrations of the growth regulators BAP, Kn and TDZ were either used alone or in combinations for the *in vitro* multiplication of shoots (Table 3). The medium with the best combinations of growth regulators was used for the multiplication of shoots. Shoots were subcultured from time to time so that number of shoots could be increased.

Table 3: Effect of different concentrations and combinations of growth regulators on *in vitro* multiplication of shoots

Media	The concentration of Growth Regulators (mg/l)		
	BAP	KIN	TDZ
MS_1	0.5	0.5	-
MS_2	1.0	0.5	-
MS_3	1.5	0.5	-
MS_4	2.0	-	0.25
MS_5	2.5	-	0.25
MS_6	3.0	-	0.25
MS_7	3.5	-	0.25
MS_8	4.0	-	0.25
MS_9	4.5	-	0.25

RESULTS

Surface Sterilization of Explants

Different sterilizing agents were used in the surface sterilization of explants of *Dendrocalamus strictus*. The sterilization was done by washing the explants under running water for about 20 minutes followed by treating explants with bavistin for 5-10 minutes. After that surface sterilization was carried out in laminar airflow with $HgCl_2$ (0.1%) and distilled water. The results of various treatments with bavistin and $HgCl_2$ are represented in the table 4.

The results show that the treatment T_2 gave the maximum percentage of survival rate of explants (83.3%), in which the treatment is given by bavistin for 10 minutes and $HgCl_2$ (0.1%) for 2 minutes. The treatment T_1 in which bavistin for 5 minutes and $HgCl_2$ for 3 minutes was least effective as only 40% culture survived.

Thus we conclude that the treatment T_2 in which $HgCl_2$ was used for 2 minutes gave the best results for the surface sterilization of the explants as compared to other treatments.

In vitro Shoot Initiation

For nodal explants, different combinations and concentrations of growth regulators in MS medium were carried out as described in the methodology. The explants were grown on MS medium supplemented with various combinations of growth regulators to establish the shoot culture. The explants were properly excised and cultured on MS medium supplemented with varying concentrations and a combination of different growth regulators. All eight media combinations were tried and consolidated results are present in table 5.

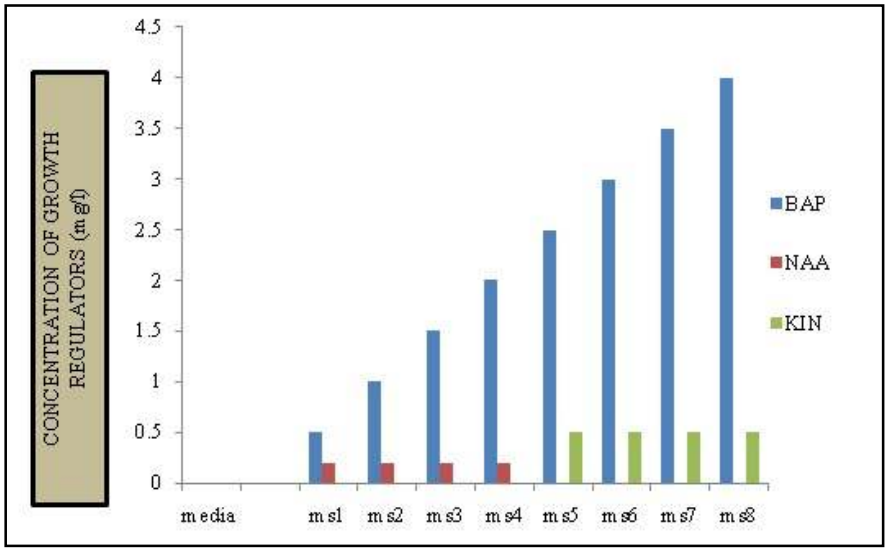
Table 4: Effect of different sterilants on percent contamination and their survival rate on explants of *Dendrocalamus strictus*

Treatments	Sterilizing agent	Quantity (w/v)	Time (minutes)	Percentage of Survival Rate	Number of explants contaminated
T_1	Bavistin $HgCl_2$	0.5% 0.1%	5 3	40%	60%
T_2	Bavistin $HgCl_2$	1.0% 0.1%	10 2	83.3%	16.7%
T_3	Bavistin $HgCl_2$	1.5% 0.1%	15 1	50%	50%

Table 5: Effect of different concentrations and combinations of growth regulators on shoot initiation of *Dendrocalamus strictus*

Media	The concentration of Growth Regulators (mg/l)			No. of days taken for shoot initiation	Remarks
	BAP	NAA	KIN		
MS_1	0.5	0.2	-	19	-
MS_2	1.0	0.2	-	15	-
MS_3	1.5	0.2	-	21	++
MS_4	2.0	0.2	-	17	-
MS_5	2.5	-	0.5	15	++
MS_6	3.0	-	0.5	22	++++
MS_7	3.5	-	0.5	21	-
MS_8	4.0	-	0.5	24	+++

++++ Maximum shoot initiation
+++ Optimal shoot initiation
++ Minimal shoot initiation



Graph 1: Concentration of Growth Regulators in Various Media

Graph 2: Number of Days taken for Shoot Initiation by Different Media

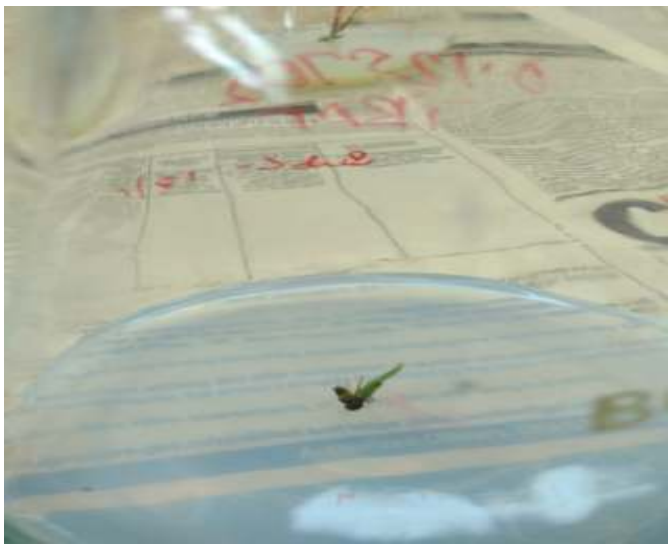


Figure 1: *In vitro* shoot initiation in MS_5 containing BAP (2.5mg/l) + Kin (0.5 mg/l) after 15 days of incubation

Figure 2: In vitro shoot initiation in MS_8 containing BAP (4.0mg/l) + Kn (0.5mg/l) after incubation of 24 days

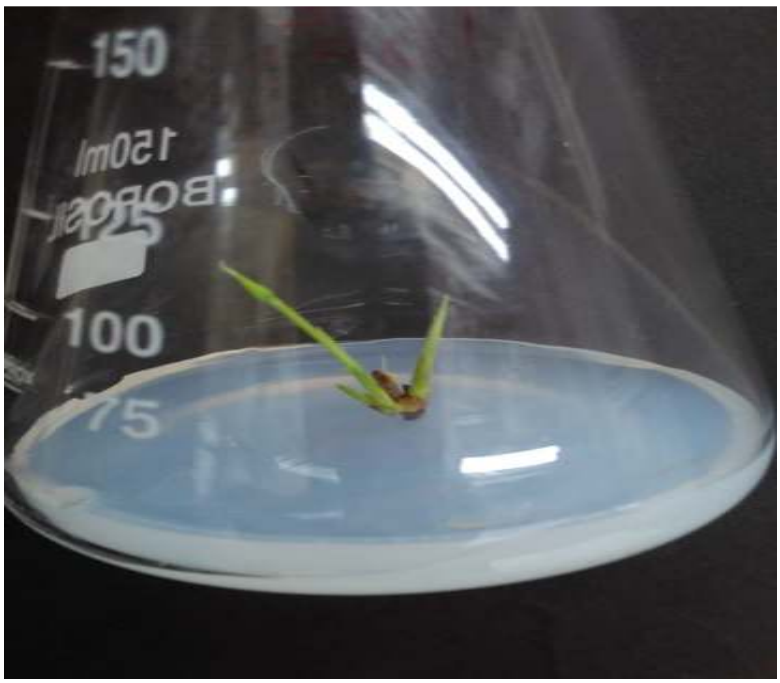


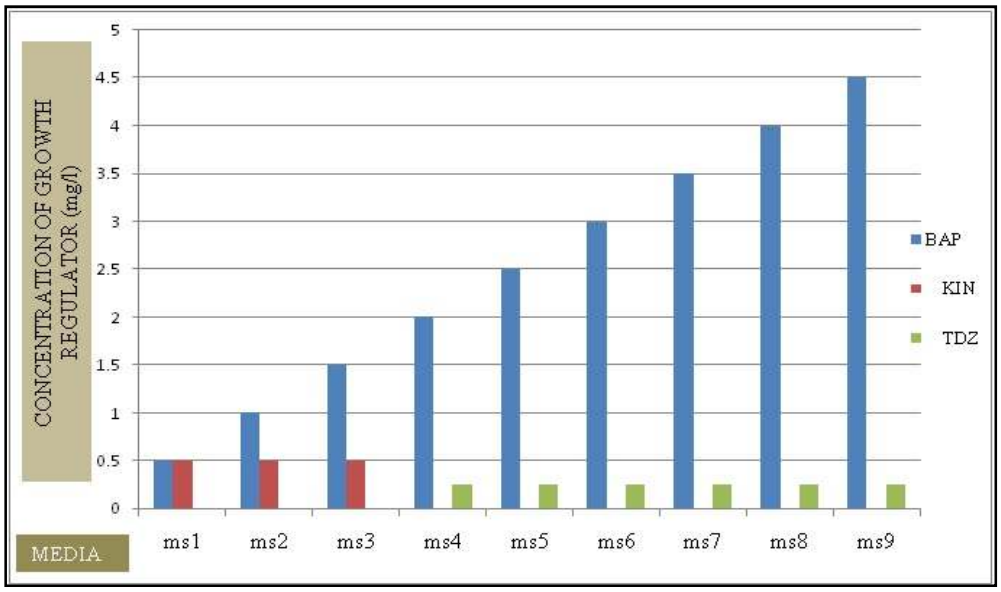
Figure 3: *In vitro* shoot initiation in MS_6 containing BAP (3.0mg/l) + Kin (0.5 mg/l) after 22days of incubation

***In vitro* Multiplication of Shoots**

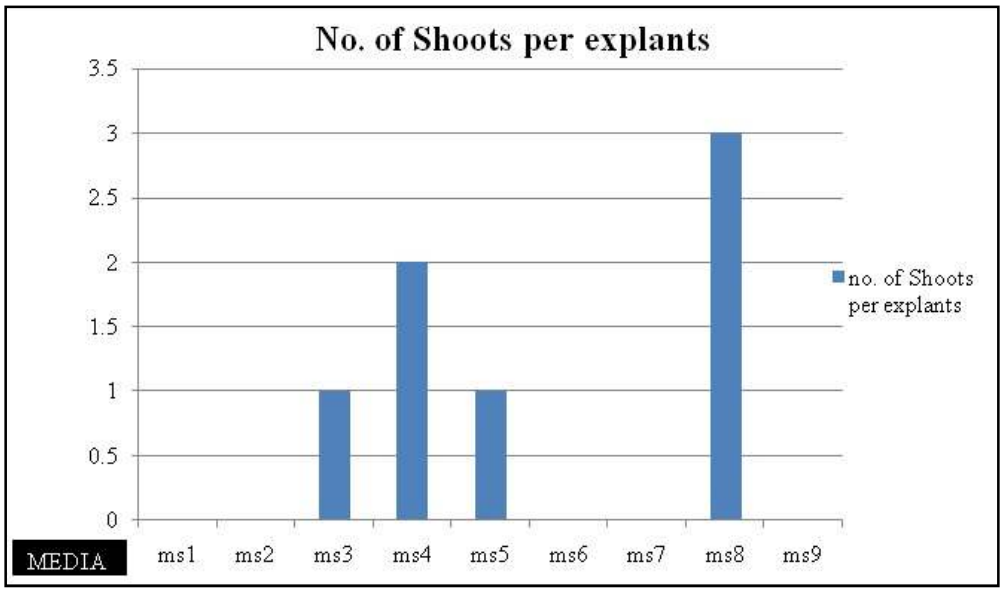
The shoots obtained from the nodal explants of *Dendrocalamus strictus* were treated with various growth regulators either alone or in combinations with one another for shoot multiplication. Major growth regulators used for shoot multiplication were BAP, Kn, and TDZ. The maximum number of shoots per nodal explants were observed in MS_8 medium containing BAP (4.0mg/l) and TDZ (0.25mg/l) while the minimum number of shoots were observed in MS_3 medium containing BAP (1.5mg/l) and Kn (0.5mg/l). Various treatments of growth regulators for *in vitro* shoot multiplication have been described in the following table 6:-

Table 6: Effect of different concentrations and combinations of growth regulators on *in vitro* multiplication of shoots

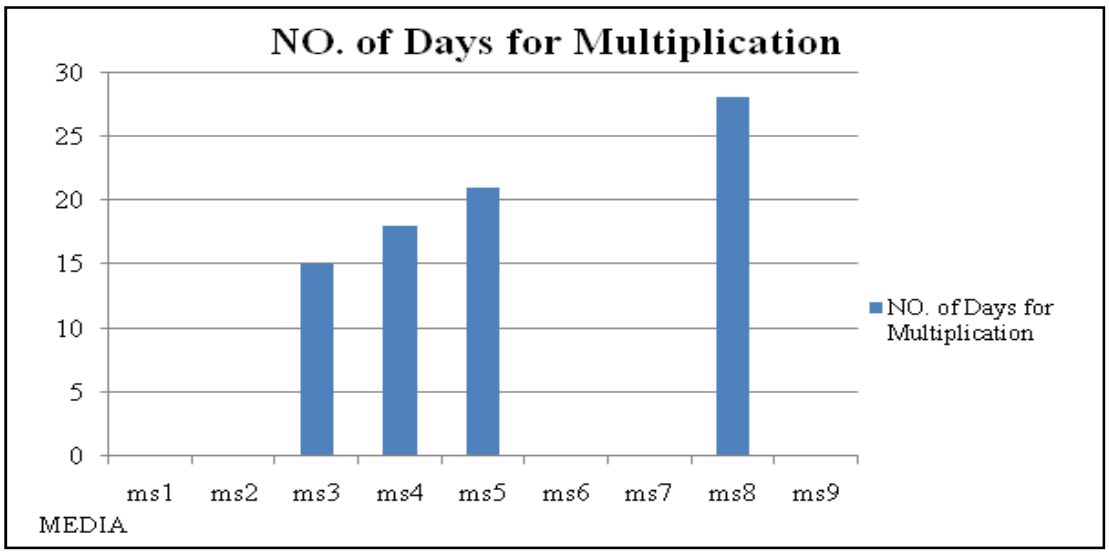
Media	The concentration of growth regulator (mg/l)			No. of shoots per explants	No. of days for multiplication
	BAP	KIN	TDZ		
MS_1	0.5	0.5	-	-	-
MS_2	1.0	0.5	-	-	-
MS_3	1.5	0.5	-	1	15
MS_4	2.0	-	0.25	2	18
MS_5	2.5	-	0.25	1	21
MS_6	3.0	-	0.25	-	-
MS_7	3.5	-	0.25	-	-
MS_8	4.0	-	0.25	3	28
MS_9	4.5	-	0.25	-	-



Graph 3: Shows the Concentration of Growth Regulators (BAP,KIN,TDZ) in different media



Graph 4: Showing the number of Shoots per explants in different Media



Graph 5: Showing Number of Days Taken for the multiplication of shoots by different Media

Figure 4: *In vitro* shoot multiplication in MS_4 containing BAP (2.0mg/l)+ TDZ (0.25mg/l)after 18 days

Figure 5: *In vitro* shoot multiplication in MS_8 containing BAP (4.0mg/l)+ TDZ (0.25mg/l) after 23 days

Figure 6: *In vitro* shoot multiplication in MS_8 containing BAP (4.0mg/l) & TDZ(0.25mg/l) after 28 days

DISCUSSION

Bamboos are among the economically most important plants worldwide and are considered as an ideal renewable resource for biomass besides their innumerable applications in handicrafts to industrial mills. An estimate regarding the future use of bamboo indicates that there will be a huge shortage of bamboo planting material in the long term and intensive research on improved propagation systems is required (Nadgauda, 1997).

In vitro Propagation of *Dendrocalamus strictus*

The present experiment was undertaken for standardization for *in vitro* shoot initiation and *in vitro* multiplication of shoots of *Dendrocalamus strictus* from selected explants.

Culture Establishment

The main purpose of culture establishment is to disinfect the explants, then establishment in culture medium and stabilize the medium and explants for multiple shoot production. For initiation of cultures, judicious selection of the explants is important because different explants tissue types within the same plant differ in their response to a particular culture condition. Nodal explants are mostly preferred due to their availability during the whole year. The suitability of nodal segments having axillary buds has been reported in micropropagation of various bamboo species like *Bambusa vulgaris* (Nadgir *et al.,* 1984; Hirimburegama and Gamage, 1995 and Ramanayake *et al.,* 2006), *Dendrocalamus strictus* (Chaturvedi *et al.,* 1993; Ravikumar *et al.,* 1998 and Mukunthakumar *et al.,* 1999), *Dendrocalamus hamiltonii* (Sood *et al.,* 1992 and Agnihotri *et al.,* 2009), etc.

Surface Sterilization of Explants

The present study was carried out by taking axillary bud as an explant from *Dendrocalamus strictus*. The collected explants were surface sterilized under running tap water for 20 minutes followed by treating explants with bavistin (1.0%) for 10 minutes and $HgCl_2$ (0.1%) for 2 minutes. Results have shown the percentage survival rate of explants i.e. 83.3%. The use of ethanol, mercuric chloride, and commercial bleach in raising aseptic cultures for control of contamination is the priority requirement in the micropropagation procedure (Huarte and Garcia, 2009; Liu *et al.*, 2011). $HgCl_2$ has proved to be the best sterilant as it is very effective in controlling pathogens (Arya *et al.,* 2008) in *Dendrocalamus asper*. Mercuric chloride was used at different concentrations of 0.2% for 25 minutes in *Dendrocalamus strictus*, *Bambusa vulgaris*, and *Dendrocalamus giganteus* (Rout and Das, 1994).

In Vitro Initiation of Shoots

During our research shoots were developed in MS medium supplemented with different concentrations of BAP, NAA, and Kn either alone or in combinations with one another to evaluate the rate of bud initiation. The best results of shoot initiation were obtained when MS medium supplemented with BAP 3.0mg/l and Kin 0.5mg/l. Ravikumar *et al.*, (1998) reported the initiation of multiple shoots in *Dendrocalamus strictus* where the MS medium was supplemented with different combinations of BAP and Kin with varying

concentrations. Chamber *et al.*, (1991) showed the bud break and multiplication in *Dendrocalamus hamiltonii* on MS medium fortified with 4.4M BAP.

In Vitro Multiplication of Shoots

It was observed that *in vitro* multiplication of shoots was obtained on the MS medium containing BAP 4.0mg/l and TDZ 0.25mg/l.

Huang L.C. and Huang B.L. (1995) reported the maximum shoot multiplication on MS medium containing BA 4.4µ

SUMMARY

The present research on *Dendrocalamus strictus* was carried by taking axillary bud as explants in the tissue culture laboratory of the Department of Biotechnology, Shoolini Institute of Life Sciences and Business Management, Solan (H.P.). The observations recorded has been summarized below:

1. The fresh, young, and disease-free axillary buds were used as explants for starting the culture.
2. The percent survival of cultures was found to be maximum (83.3%%) with the treatment Bavistin (1.0%) for 10 minutes and $HgCl_2$ (0.1%) for 2 minutes. Thus this was the best treatment for sterilization as it produced a maximum percentage of uncontaminated cultures.
3. Maximum shoot initiation occurred when MS medium containing BAP 3.0mg/l and Kn 0.5mg/l were used.
4. Maximum shoot multiplication occurred when MS medium containing BAP 4.0mg/l and TDZ 0.25mg/l were used.

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