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Rapid Shoot Proliferation of Woodfordia fruiticosa (L) Kurz in Liquid Shake Culture System*

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ABSTRACT

Woodfordia fruiticosa (L) Kurz is a plant of tropical and subtropical region with a long history of medicinal use. The present investigation reveals that the highest proliferation of shoots was observed in liquid M S (Murashige Skoog) medium under shaking condition. Shoot initiation, multiplication in semisolid media and shoot proliferation in liquid media in both stationary as well as shaking conditions were performed. After 15 days of incubation, hormone free plain MS media was recorded as the best media for shoot initiation which gave maximum no of shoots with larger shoot length, it was 3.7±1.33 (shoot no.) and 2.46 ±1.06 (shoot length). Semi solid medium selected for shoot multiplication was MS medium supplemented with 0.5mg/l BAP (benzyl amino purine), whose maximum no of shoots with shoot length was recorded as 4.35 ± 1.69 (shoot no.) and 12.34 ± 3.52 (shoot length). On sub-culturing in liquid MS medium with 0.5mg/l BAP in stationary phase, after two weeks it was observed that the rate of shoot proliferation was faster than semisolid medium which produced maximal number of shoots with elongation, it was 7.12±1.78 (shoot no.) and 14.62±1.37 (shoot length). Further, shoots in liquid MS medium with 0.5mg/l BAP kept in shakers proliferated tremendously than those kept in stationary phase and propagated highest number of shoot with enormous shoot length, it was 17.5 ± 2 (shoot no.) and 11.5±8.92 (shoot length).

Keywords: BAP, B5 medium, kinetin, liquid shake culture system, Murashige Skoog, shoot nodes, Woodfordia fruticosa.

I. INTRODUCTION

Woodfordia fruticosa (L) Kurz is plant of tropical and subtropical region with a long history of medicinal use. It is popularly known as Dhataki which is generally used in Ayurvedic medicine. *Woodfordia fruticosa* (L). Kurz (family Lythraceae) is a rare woody shrub distributed up to an altitude of 1500 km in the hilly tracts of northern India mainly found in Madagascar, India, Pakistan, Ceylon, China, Indonesia, throughout India but abundantly found in north India up to 1600 m widespread in the foothills [1]. Almost all the parts of plants have medicinal properties, but there is high demand for its flowers. A wide range of chemical compounds including tannins, flavonoids, anthrax quinone glycosides and polyphenols have been isolated from this species in recent times. Extracts and metabolites of this plant, particularly those from flowers and leaves, possess useful pharmacological activities [2]. The red dye extracted from the flowers is extensively used throughout India for dyeing fabrics [3].

Nowadays there is a great demand for medicinal plants with higher growth potential. Propagation via seed is difficult in this species [4]as well as vegetative propagation is also very difficult). Due to all these reasons and

medicinal importance of it, formulation of a standard, rapid shoot proliferation protocol for *W. fruticosa* is important. A rapid propagation method comprising initiation of in vitro shoot tip culture from field grown flowering plants and reculture of nodal segments of regenerated shoots in Schenk and Hildebrandt 1972 medium was developed for *Woodfordia fruticosa* (L). Kurz., a rare medicinal shrub [5].

An efficient rapid *in vitro* regeneration protocol was developed for *Woodfordia fruticosa* (Linn.) Kurz., a wild threatened medicinal ornamental shrub, which is in need to be conserved, by tissue culture techniques. The plants were regenerated from young nodal segments and shoot tips. Shoot tips were the best explants for direct organogenesis and nodal segments were used as explants for indirect organogenesis. Maximum number of multiple shoots was obtained from nodal segment explants on MS medium supplemented with (0. 2-2. 5 mg/l) BAP with 95% shoot regeneration response. Maximum shoot height of 9-11 cm was achieved. This is a rapid, reproducible protocol for large scale propagation of this rare, much- used medicinal, beautiful, ornamental, threatened plant species and its ex-situ conservation [6].

Micro propagation by conventional techniques is typically a labor intensive time taking means of clonal propagation. Hence, the most challenging aspect at present is to reduce the production cost, thereby improving the production efficiency. Nowadays the use of shake cultures utilizing liquid culture medium alone or in combination with solid culture medium have been developed and used by various workers.

Therefore the present investigation was designed to study the rapid shoot proliferation protocol of *woodfordia fruticosa* through nodal portion in liquid media in both stationary phase and in shaking incubators.

II. MATERIALS AND METHODS

2.1 Plant material

For *in vitro* regeneration of *Woodfordia fruticosa* newly grown young healthy nodal portion were selected as explants. Young healthy shoot nodes were taken from plants of *Woodfordia fruticosa*, which were grown in the botanical garden of School Of Studies in Life Sciences, Pt. Ravishankar Shukla University, Raipur (C.G.).

2.2 Isolation and Excision of explants

Young healthy nodal portions were carefully removed from the plants with the help of sterilized blade in aseptic conditions then internodes were cut from both the upper and lower parts in such a way that the middle part contains the shoot nodes.

2.3 Sterilization of explants

The explants (young shoot nodes) were washed with tween -20 (liquid detergent) and later washed with running tap water. The explants were then washed thoroughly with distilled water after this the surface sterilization was performed inside the laminar air flow hood. For standardization of surface sterilization, explants were given five different treatments with 0.1 % HgCl2, varying the time from 1min to 5 min. Each time after the treatment with 0.1% HgCl2 they were washed with double distilled water for 3-4 times and were dipped in double distilled water until the inoculation.

2.4 Initiation of *in-vitro* culture for rapid shoot proliferation

Initiation of *in-vitro* culture is an important step which requires standardization of suitable medium. For this different media like plain Murashige Skoog (MS) media and MS media with three different concentration of hormone, 6-benzylaminopurine (BAP) (MS+0.5 mg/l BAP, MS+1mg/l BAP, MS+2mg/l BAP) and also with three combination of Kinetin (Kin.) (MS+0.5mg/l BAP+ 0.5mg/l Kin., MS+0.5mg/l BAP+ 1mg/l Kin, MS+0.5mg/l BAP+ 4 mg/l Kin), were used. B5 medium, Schenk Hildebrandt Medium (SH) and wood plant media (WPM) each with 0.5 mg/l BAP were also used for selection of shoot initiation media.

After initiation, the plantlets were sub-cultured first in semi-solid MS media containing 0.5 mg/l BAP, 0.5 mg/l BAP+0.5 mg/l Kin, 1mg/l BAP+0.5 mg/l NAA and 0.5 mg/l BAP+ 1 mg/l NAA. and visual observation was taken after every 15 days.

Similarly, fresh explants from the initial culture were inoculated in liquid MS media containing 0.5 mg/l BAP, 0.5 mg/l BAP+0.5 mg/l Kin with the help of sterilized forceps. The cultures in liquid media were incubated separately in stationary phase and in shaking incubator at 90 rpm and 25 ºC. Cultures from liquid medium were further transferred in ½ MS medium, and the MS medium containing 0.5 mg/l IBA and 1mg/l IBA for rooting. Afterwards, the cultures were observed and the results were recorded at the intervals of every fifteen days and a comparative data analysis was done statistically between the shoots regenerated from the semisolid and liquid media kept in both stationary and in shaking phase in terms of average ± standard deviation (SD).

III. RESULT AND DISCUSSION

The explants sterilized with 0.1% $HgCl_2$ for 5 min was standardized for surface sterilization (table no. 1 and fig 7) which gave best initiation in plain (hormone free) MS media as tabulated in table No. 2 according to which hormone free plain MS media was resulted out to be the best media for shoot initiation which gave higher no of shoots with larger shoot length, it **was 3.7±1.33 (shoot no mean ± SD) and 2.46±1.06 (shoot length mean ± SD)** which was also explained graphically in fig 8 and fig 9.

In this work the explants (nodal region of shoots) were first surface sterilized with 0.1% mercuric chloride for one to five minutes. Best sterilization was achieved in five minute treatment with 100% response without contamination. Similar result was observed by Gayathri *et al.,* 2000 [7]

The investigation was undertaken to develop techniques for rapid regeneration of *W. fruticosa*. The success of tissue culture experiments depend upon the composition of culture medium therefore according to this investigation plain MS medium resulted out to be the best media for the initiation of cultures (Fig. 1and 2).

After shoot initiation well initiated shoots were transferred to other medium for shoot multiplication in which MS media subjected with different concentration of hormones *viz.* BAP (0.5mg/l BAP) and combination of BAP and Kinetin (0.5mg/l BAP+0.5mg/ Kin) and NAA (0.5mg/l BAP+1mg/l NAA, 1mg/l BAP+0.5mg/l NAA). According to table no.3 best result was observed in 0.5 mg/ml BAP only which produced maximum no of shoots (fig 3 and 4), it was **4.35 ± 1.69** (shoot no. mean ± SD), **12.34 ± 3.52** (shoot length mean ± SD) also presented graphically in fig 10 and fig 11 . Finding are partial agreement to the study of Islam *et al.,* 2009[8] cultured *Woodfordia fruticosa* in MS supplemented with BAP (0.5 - 3.0 mg/l and Kn (0.5 - 1.0 mg/l). According to him large numbers of multiple shoots with aerial tiny roots were produced in the culture medium containing

BAP. Similarly Krishnan *et al.*,1994 [5] also reported a medium supplemented with 6- BAP (0.2 mg/l) induced high frequency (88%) development of axillary shoot buds (3.2 cm) in 4-5 weeks. He reported highest multiplication of shoots (26-35 shoots) using culture initiation medium with 0.5 mg/l each of BAP and NAA followed by subculture in 0.2 mg/l BAP.

Shoot multiplication not much improved in semisolid medium after a couple of subcultures. Hence, for further proliferation, multiplication was performed in hormone free liquid MS media and liquid MS media supplemented with 0.5mg/l BAP+0.5mg/l Kin, 0.5mg/l BAP medium in stationary phase and in shakers. In stationary phase, it was resulted out that the maximum number of shoot buds with elongation was produced recorded as **7.12±1.78** (shoot no. mean ± SD) and **14.62±1.37** (shoot length mean ± SD) as tabled in table no.4 also represented graphically in fig 12 and fig 13. Shoot proliferation tremendously improved under shaking condition in liquid MS medium with 0.5mg/l BAP, which was recorded as **17.5 ± 2** (shoot no. mean **± SD**) and 11.5±8.92 (shoot length mean **± SD**) the results were higher as compare to those which obtained in liquid medium under stationary phase (table no. 5 and fig 5and 6) represented graphically in fig 14 and fig 15.

The findings were comparable with the study performed by Zuraida *et al.,* 2011 [9] in which pineapple plants were cultured on MS liquid medium supplemented with 1 mg/l BAP produced the highest number of shoots (31) after 4 weeks. The number of shoots formed was increased to 204 after third sub-culture in liquid medium. Shoot proliferation was increased up to nine fold in liquid medium when compared to the cultures maintained on solid medium. Similarly, Tefsa *et al.*,2016 [10] reported in vitro shoot multiplication of elite sugarcane genotypes i.e., N52 and N53 in which they resulted out that N52 showed a maximum of 6.95 ± 0.19 shoots per explant with 4.75 ± 0.06 cm shoot length and 5.65 leaves per shoot on liquid MS medium amended with 2 mg/l BAP + 0.5mg/l kinetin while genotype N53 produced a maximum of 6.30 ± 0.26 shoots per explant with 3.94 ± 0.03 average shoot length and 5.83 leaves per shoots on liquid MS medium amended with 1.5 mg/l BAP + 0.5 mg/l kinetin.

The liquid medium allows the close contact with the tissue which stimulates and facilitates the uptake of nutrients and phytohormones, leading to better shoot and root growth. Continuous shaking promotes lesser expression of apical dominance which generally leads to induction and proliferation of numerous axillary buds. Further, with in the shake culture conditions, the growth and multiplication rate of shoots is enhanced by forced aeration, since continuous shaking of medium provides ample oxygen supply to the tissue which ultimately leads to their faster growth. This leads to the development of bud clusters which are amenable to the control of medium components, to mechanical separation and to automated inoculation as an efficient delivery system to the final stage for plant growth [11].

Teklehaymanot et al. 2010 [12]compared between three *In vitro* systems i.e., solid media, shaking liquid media, temporary immersion system for obtaining the highest shoot proliferation. They compared the effect of 2.5 or 5 µM Meta-topolin (m T) with or without 2.5µM NAA with the MS basal medium. After six weeks, the medium with 2.5µM m T led to the highest shoot proliferation in shaking liquid system.

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IV. FIGURES AND TABLES

4.1 FIGURES

Fig 1

Fig 2

Fig 1 and 2. Initiation in plain MS medium

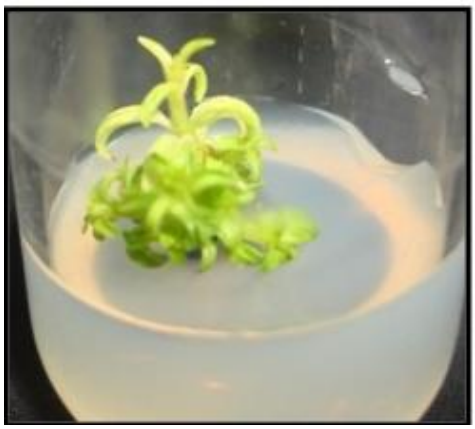

Fig 3.

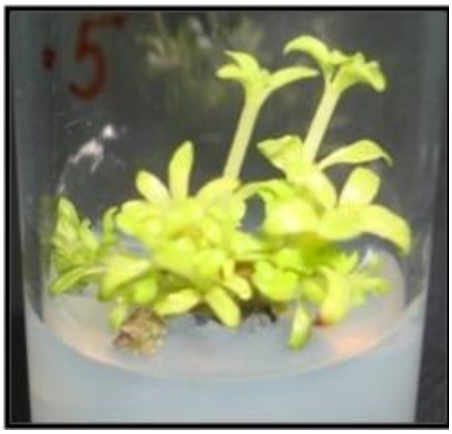

Fig 4

Fig 3 and 4 showing subculturing and shoot multiplication in semisolid medium.(MS+0.5BAP)

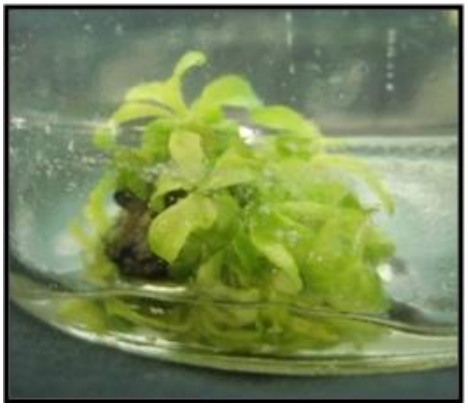

Fig 5

Fig 6

Fig 5 and 6 showing shoot multiplication in liquid medis(MS+0.5BAP)
in stationary phase and in shaker respectively.

4.2. Tables

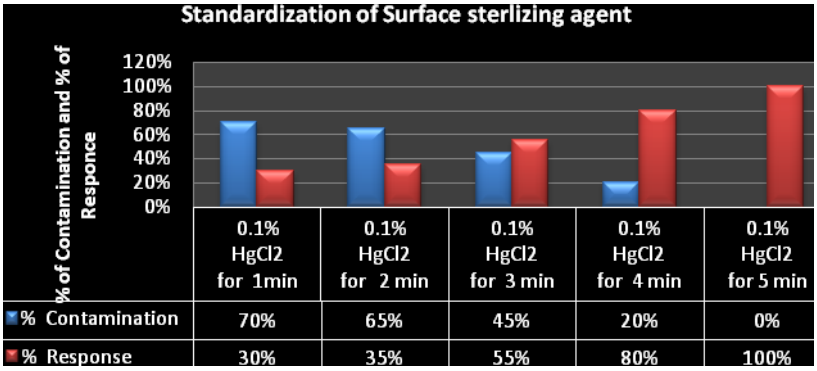

Fig 7

Fig 7 showing standardization of surface sterilizing agent

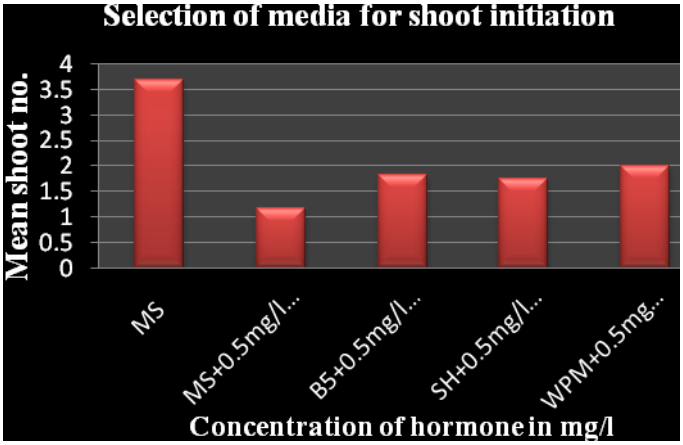

Fig 8

Fig 8 showing selection of media for shoot initiation (shoot No.)

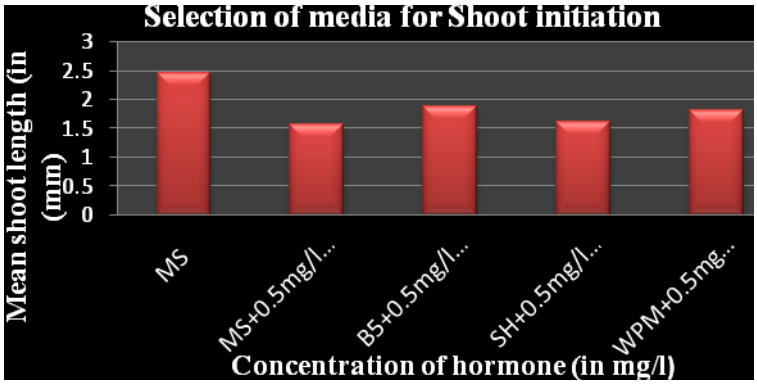

fig 9

Fig 9.showing selection of media for shoot initiation (shoot length)

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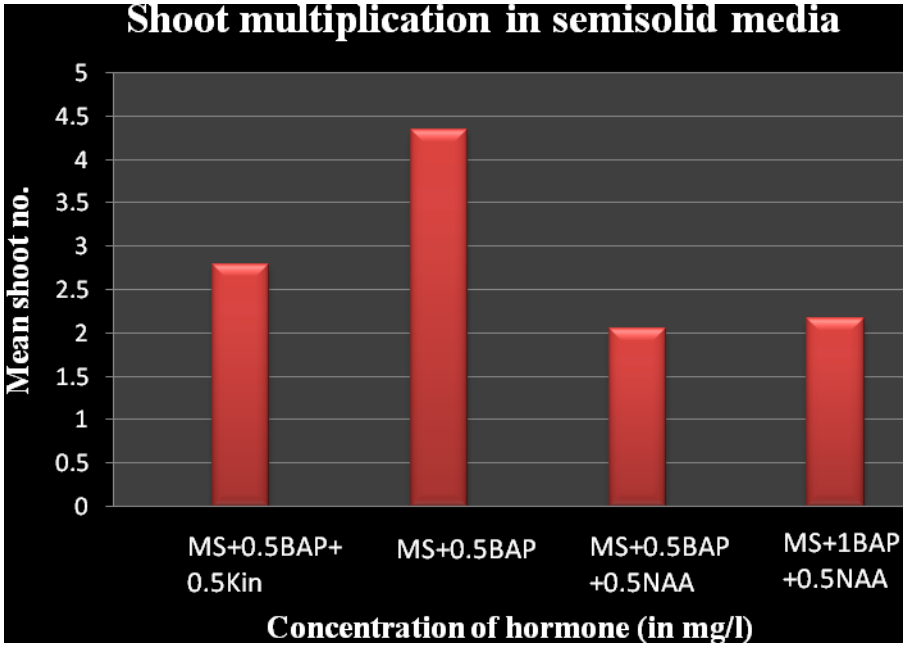

fig 10

fig 10 shoot multiplication in semi solid media (shoot No.)

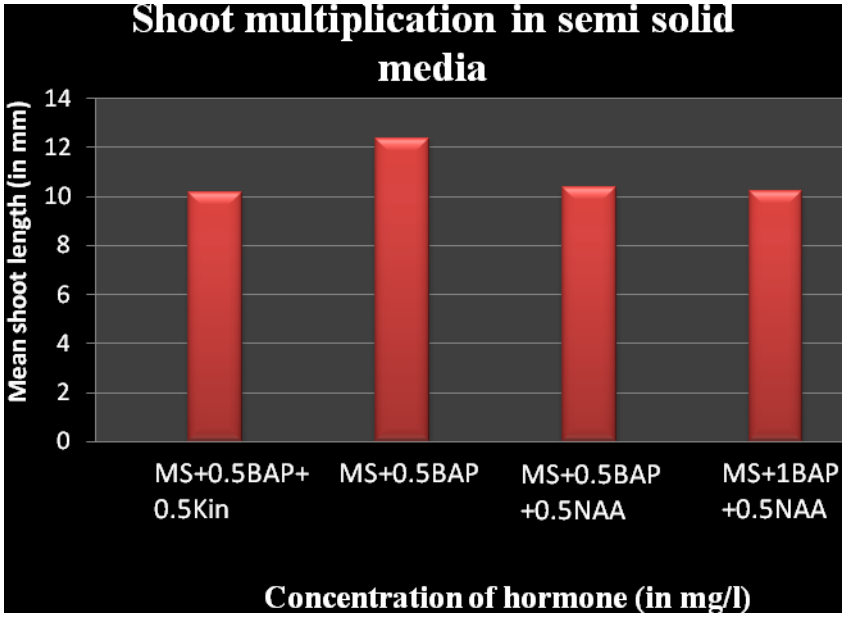

fig 11

fig 11 Shoot Multiplication in Semi solid media (shoot length).

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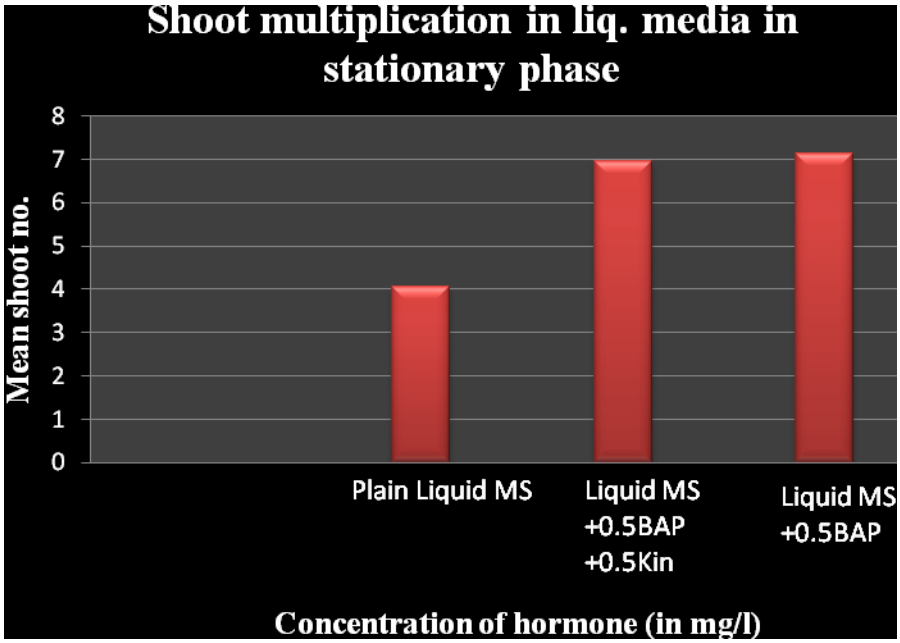

fig 12

fig 12 shoot multiplication in liquid media in stationary phase (Shoot no)

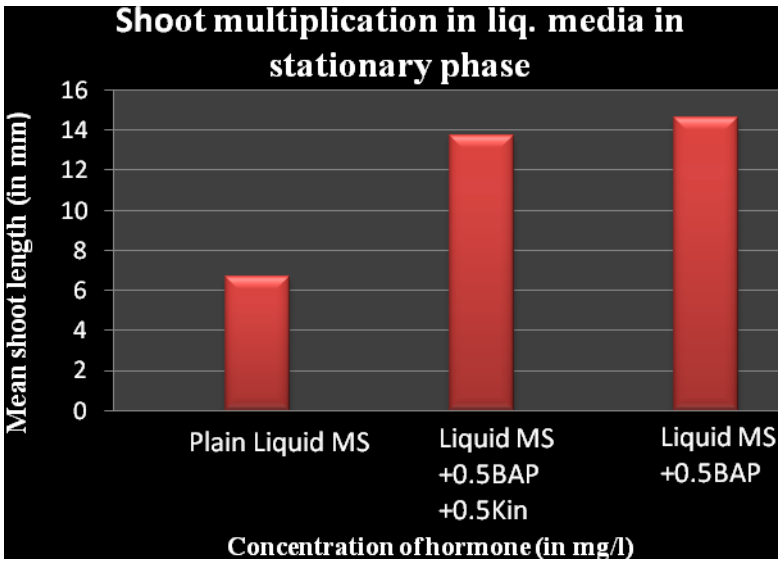

Fig 13

Fig 13 shoot multiplication in liquid media in stationary phase (Shoot length)

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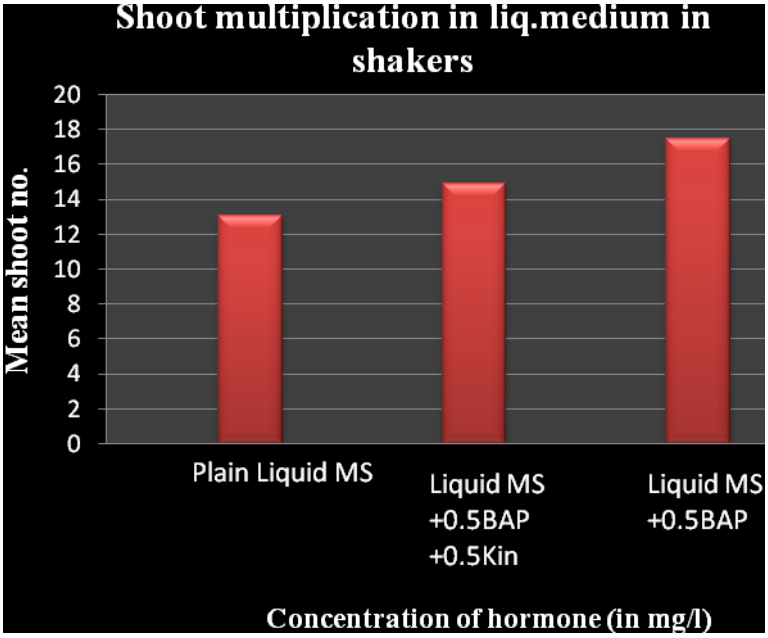

Fig 14

fig 14 shoot multiplication in liquid media in shaking phase (Shoot No.)

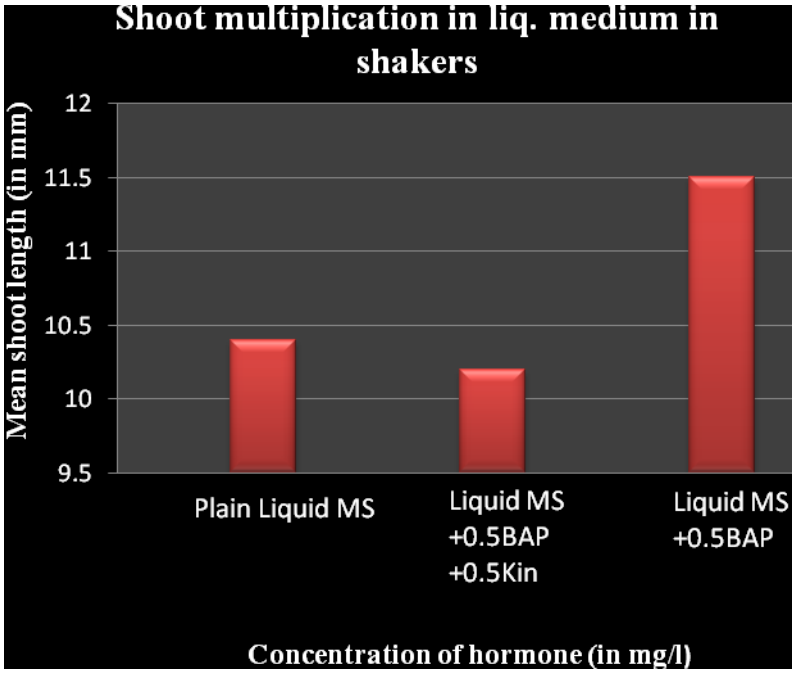

fig 15

fig 15 shoot multiplication in liquid media in shaking phase (Shoot length)

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4.2 Tables

Table1. Standardization of surface sterilizing agents:

S. No.	Treatment	Time Duration	% contamination	% response
1	0.1% HgCl2	1min	70%	30%
2	0.1% HgCl2	2 min	65%	35%
3	0.1% HgCl2	3 min	45%	55%
4	0.1% HgCl2	4 min	20%	80%
5	0.1% HgCl2	5 min	0%	100%

Table No. 2: Selection of media and concentration of hormone for culture initiation:

S. No.	Medium	Concentration of Hormone (mg/l) BAP + Kin		Shoot no. (mean ± SD)	Shoot length(mm) mean ± SD
1	MS	-	-	3.7 ± 1.33	2.46 ± 1.06
2	MS	0.5	-	1.16 ± 0.40	1.58 ± 0/49
3	MS	1.0	-	1.50 ± 0.50	1.25 ± 0.25
4	MS	2.0	-	1.33 ± 0.47	1.66 ± 0.57
5	MS	0.5	0.5	1.87 ± 0.83	1.81 ± 0.74
6	MS	0.5	1.0	1.66 ± 0.81	1.71 ± 0.77
7	MS	0.5	4.0	1.75 ± 0.43	1.5 ± 0.70
8	B5	0.5	-	1.83 ± 0.75	1.88 ± 0.56
9	SH	0.5	-	1.75 (.46	1.62 ± 0.74
10	WPM	0.5	-	2.00 ± 0.75	1.81 ± 0.84

note: MS-Murashige and Skoog; SH- Schenk Hildebrandt; WPM- wood plant media; BAP-benzyl amino purine; Kin- kinetin. SD- Standard deviation

Table 3. Shoot multiplication in semisolid media amended with BAP, Kinetin and NAA

S. No.	Media	Concentration of hormone(mg/l)			Shoot no. (mean ± SD)	Shoot length (mm) mean ± SD
		BAP	**Kin**	**NAA**		
1	MS	0.5	-	-	**4.35 ± 1.69**	**12.34 ± 3.52**
2.	MS	0.5	0.5	-	2.80 ± 2.63	10.17 ± 5.59
3.	MS	0.5	-	1	2.06 ± 1.39	12.35 ± 9.07
4	MS	1.0	-	0.5	2.17 ± 1.30	10.23 ± 3.53

note: MS-Murashige and Skoog; BAP-benzyl amino purine; Kin- kinetin; NAA- Napthelene acetic acid; SD- Standard deviation

Table 4. Shoot multiplication in liquid media in stationary phase

S. No.	Media	Concentration of hormone (mg/l)		Shoot no.	Shoot length(mm)
		BAP	Kin	mean± SD	± SD
1	Liquid MS	-	-	4.06 ± 0.25	6.7 ± 2.19
2	Liquid MS	0.5	0.5	6.95 ± 0.95	13.77 ± 2.59
3	Liquid MS	0.5	-	**7.12 ± 1.78**	**14.62 ± 1.37**

note: MS-Murashige and Skoog; BAP-benzyl amino purine; Kin- kinetin; SD- Standard deviation

Table 5. Shoot multiplication in liquid media in shaking incubator

S. No.	Media	Concentration of hormone (mg/l)		Shoot no.	Mean Shoot length
		BAP	Kin	mean ± SD	(mm) ± SD
1	Liquid MS	-	-	13.1 ±2 .75	10.4 ± 3.12
2	Liquid MS	0.5	0.5	14.9 ± 2.57	10.2 ± 2.45
3	Liquid MS	0.5	0.5	17.5 ± 2.00	11.5 ± 8.92

Note: MS-Murashige and Skoog; BAP-benzyl amino purine; Kin- kinetin; SD- Standard deviation

V. ACKNOWLEDGEMENT

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