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In vitro Regeneration of *Catharanthus roseus* and *Bacopa monnieri* and their Survey around Jaipur District

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ABSTRACT

An efficient and cost effective micro propagation protocol using MS medium was developed for Catharanthus roseus and Bacopa monnieri. Shootlets were regenerated from nodal explants of stem through auxiliary shoot proliferation.

In Catharanthus roseus, the induction of multiple shoots from nodal segments were premier in MS medium supplemented with 0.5 mg/l BAP ± 1mg/l NAA. and maximum rooting was recorded on MS medium with 5.0 mg/l IBA.

In Bacopa monnieri, the induction of multiple shoots were highest in MS medium supplemented with 0.5 mg/l BAP + 2.0 mg/l Kn and 0.5 mg/l Kn+1.0 mg/l BAP. and highest rooting was recorded on MS medium supplemented with 2.0 mg/l IBA. Leaf petiole explants were used for the purpose of callus induction. Best growth was observed in MS medium supplemented with 0.25 mg/l 2, 4-D+ 0.5 mg/l Kn and 0.25 mg/l 2,4-D+ 0.1mg/l BAP.

Key words: *Catharanthus roseus, Micropropagation, Medicinal plant, Callus induction.*

INTRODUCTION

Herbal medicine have always occupied the nuclear place in every traditional system of medicine.It may be *Ayurveda, Unani* or *Sidtha*. More than 21,000 plants possess medicinal values which have been listed by World Health Organization (WHO). The use of plants as medicines has a long history in the treatment of various diseases. The earliest known records for the use of plants as drugs are from Mesopotamia in 2600 B.C[8]. *In vitro* regeneration or micropropagation has tremendous potential for rapid multiplication and production of high quality medicinal plants [17]. *Catharanthus roseus* (L.) G. Don. (Commonly known as *sadabahar* or Periwinkle, family apocynaceae), gained commercial importance because of its medicinal properties. It is an erect handsome herbaceous perennial plant which is a chief source of patented cancer and hypotensive drugs. *Catharanthus roseus* has more than 400 known alkaloids in its different parts. The alkaloids like antineoplastindimeric, vinblastin and vincristine are mainly present in aerial parts, whereas ajmalcine, vinceine, vincamine, raubasin and reserpine are present in roots and basal stem. The dimericindole alkaloids from *C.roseus* are mainly used for treatment of various human cancers. Pharmaceutical industry used it for the treatment of childhood leukemia, Hodgkin's disease, testicular cancer and cancerous tumors. *C.roseus* is one of the very few medicinal plants which have a long history of uses as diuretic, antidysenteric, hemorrhagic and antiseptic. It is known for use in the treatment of diabetes in Jamaica and India. Prevention of cancer, cancer treatment, anti-diabetic, stomachic, reduces high blood pressure, externally against nose bleeding, sore throat and mouth ulcers.

In *C.roseus*, node culture producing shoots were noticed [4-7]. Optimization of conditions for shoot multiplication has been attempted for continuous production of useful compounds without depletion of natural flora [7,26,13,15]. Selection of suitable experiments depends on various factors such as physiological stage, type of organ tissue, seasonal variations of parent plant from which the explants is selected [16].

In present study, attempts were made for *in vitro* shoot regeneration of *Catharanthus roseus* using different explants. These efforts are made to study plant regeneration using shoot tip, hypocotyls and epicotyls explants, this have done in light conditions.

Bacopa monnieri (L.), commonly known as ''Brahmi'', is a member of the Family Scrophulariaceae, is placed second in the priority list of Indian medicinal plants . It is commonly found on the banks of rivers and lakes. It has been used for centuries in folklore and traditional system of medicine as a memory enhancer, anti-inflammatory, analgesic, antipyretic, sedative and anti-epileptic agent. The memory enhancing effects of *Bacopa monnieri* have been attributed to the active constituent bacosides A and B. In addition to its unique medicinal use, *Bacopa monnieri* has also been linked to phytoremediation programmes for the removal of heavy metals such as cadmium and chromium.

In 1990, the annual requirement of this plant was 12.7 × 106 kg of dry biomass at a value of $34 million . With increasing demand for herbal drugs, the natural populations of *Bacopa monnieri* are threatened with overexploitation. So The International Union for Conservation of Natural and National Resources has a long time ago listed *Bacopa monnieri* as a threatened species. There is a demand for further improvement in the tissue culture protocol for the mass multiplication of *Bacopa monnieri*, both for commercial farming system and later, if required for replanting in the natural habitat when the plant population declines. We have developed an innovative micropropagation protocol that has not been attempted so far in *Bacopa monnieri*.

The aim of the present study was to develop high frequency multiple shoots regeneration of *Bacopa monnieri* utilizing the least number and various concentrations of PGRs. This protocol also offers the rapid callus formation from the leaf petiole. For both purposes Auxin (2, 4-D) and cytokine (kn., BAP) are used.

MATERIAL AND METHODS

The fruits of *Croseus and Bacopa monnieri*. were collected from villages of Jaipur District of Rajasthan. They were dried. The seeds were surface sterilized subsequently with running tap water, labolin soap solution and finally with 0.1% $HgCl_2$ for 15 min under laminar bench. After rinsing for 5-6 times with autoclaved distilled water, they were inoculated aseptically on to water agar (0.8%) for germination. Cotyledonary and epicotyledonary nodes obtained from 15-d-old seedlings were implanted vertically on to different culture media [Murashige and Skoog (MS)] containing 2.0 mg/l BAP. Different concentrations of BAP and Kn (1.0-5.0 mg/l) were used individually for proliferation of shoots from seedling nodes. Combination of BAP and Kn were used for shoot proliferation. Same experiments were repeated for shoot multiplication.

The medium containing 3% sucrose was solidified with 0.8% agar (Qualigens). The *p*H of the media was adjusted to 5.9+0.02 with 1 N NaOH or 1 N HCl solutions prior to autoclaving. Media poured in culture vessels were steam sterilized by autoclaving at 15 lbs for 15-20 min. The cultures were incubated under controlled conditions of temperature (25±2°C), light (2000-2500 lux for 16 h/d provided by fluorescent tubes) and 60-70% humidity. For each experiment a minimum of 7 replicates were taken and experiments were repeated thrice. Observations were recorded after an interval of 3 wk. Once culture conditions for shoot induction from explants were established, the shoots produced *in vitro* were sub cultured on fresh medium every 3 wk. Different combination of Auxins (0.25 mg/l) (2, 4-D) and Cytokinine (BAP and Kn) (0.1-0.3 mg/l) were used for callus induction from leaf petiole. Rooting of elongated shoots was attempted under *in vitro* conditions. Auxins (IBA) alone in different concentrations (0.5-2.5mg/l) were incorporated in the agar (0.8%) solidified medium containing 1/4 MS salts and 1.0% sucrose. The *in vitro*-rooted plantlets were transferred to culture bottles 1/4th filled with Soilrite composition.

RESULTS AND DISCUSSION FOR *Catharanthus roseus*

The nodal explants, when inoculated on MS medium containing single cytokinin(BAP , Kn)and auxin(NAA,IAA) in the range 1.0-5.0 mg/l showed enhanced shoot proliferation. BAP at its 1.0 mg/l concentration evoked best response (Table-1, 2Figure-1 A, B, Figure-2,4).

Shoots after their initial proliferation on medium containing 1.0 mg/l BAP were sub-cultured on same fresh medium after every 21 days. Incorporation of single BAP , Kn, NAA and IAA into MS medium supported multiplication of shoots in culture, BAP proved to be a better choice than other and the maximum number of shoots were obtained on its 1.0 mg/l concentration (Table-1,2 Figure-1 A, B, Figure-2,4).

When BAP was used in combination with NAA a variety of responses were observed (Table-3, Figure-1 C, D and Figure-3). But best response was observed on medium containing 0.5 mg/l BAP + 1.0 mg/l NAA (Average number of shoots (7.30±0.64) and Average shoot length (5.97±0.17cm).

The full or half strength of MS medium without any PGR was failed to induce rooting of regenerated shoots. However, shoots were capable to induce root when cultured on medium containing auxins.

IBA in different concentrations induced rooting when incorporated in the medium containing ¼ of MS salts. The best rooting response, however, was observed on medium containing 5mg/l IBA, where roots measuring 2.80±0.73 cm (average) were formed (Table-4, Figure-1 E, Figure- 5).

In vitro rooted plantlets were initially hardened in culture room conditions where leaves expanded. After 3 weeks, the plantlets were shifted to mist house. There was an increase in length of shoots and new leaves emerged which expanded quickly (Figure-E).

Table-1: Effect of cytokinin (BAP and Kn) on shoot proliferation from nodal shoot explant of *Catharanthus roseus*

Hormone Concentration (mg/ l)	Hormone Concentration (mg/ l)	Response (%)	Number of Shoots/explant (mean±SD)	Shoot length (in cm) (mean±SD)
BAP	**Kn**			
1.0	-	98	7.12±0.45	1.80±0.28
2.0	-	92	5.40±0.81	2.54±0.65
3.0	-	86	3.40±0.24	1.36±0.74
4.0	-	72	2.30 ±0.31	1.19±0.21
5.0	-	70	1.73±0.87	1.00±0.15
	1.0	97	6.67±1.22	2.70±1.50
	2.0	80	5.80±0.24	2.50±0.94
	3.0	96	3.87±0.39	3.36±0.29
	4.0	76	4.50±0.20	2.23±0.11
	5.0	72	3.31±0.30	1.27±0.85

Medium: MS+ additives; mean± SD, n= 7 replicates,

Means having the same letter in each Colum do not different significantly at P< 0.05 (Tukey's test)

Table-2:Effect of Auxin (NAA and IAA) on shoot proliferation from nodal shoot explant of *Catharanthus roseus*

Hormone Con. (mg/ l)	Hormone Con. (mg/ l)	Response (%)	No. of Shoot/explant (mean±SD)	Shoot length (in cm) (mean±SD)
NAA	**IAA**			
1.0	-	87	5.23±0.12	1.77±0.39
2.0	-	60	4.40±0.94	2.15±0.28
3.0	-	75	5.75±0.34	3.31±0.30
4.0	-	70	5.80±0.24	2.50±0.94
5.0	-	65	3.95±0.03	1.65±0.65
-	1.0	70	1.57±0.40	1.62±0.28
-	2.0	68	2.42±0.39	2.28±0.71
-	3.0	75	2.28±0.36	0.53±0.33
-	4.0	55	1.08±0.19	0.28±0.13
-	5.0	30	1.28±0.36	0.15±0.18

Medium: MS+ additives; mean± SD, n= 7 replicates

Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table-3:Interactive effect of cytokine (BAP+ NAA) on shoot multiplication by sub culture of shoot Clumps of *Catharanthus roseus*

Hormone Concentration (mg/ l)	Number of Shoots/explant	Shoot length (in cm)	Shooting Response (%)
0.5 BAP + 0.5 NAA	6.50±0.27	3.87±0.39	98
0.5 BAP + 1.0 NAA	7.30±0.64	5.97±0.17	99
0.5 BAP + 2.0 NAA	5.02±0.76	3.06±0.22	97
0.5 BAP + 2.5 NAA	4.98±0.74	2.31±0.48	82
0.5BAP + 3.0 NAA	3.78 ±0.57	2.17±0.47	78

Medium: MS+ additives; mean± SD, n= 7 replicates

Means having the same letter in each Column, do not different significantly at P< 0.05 (Tukey's test)

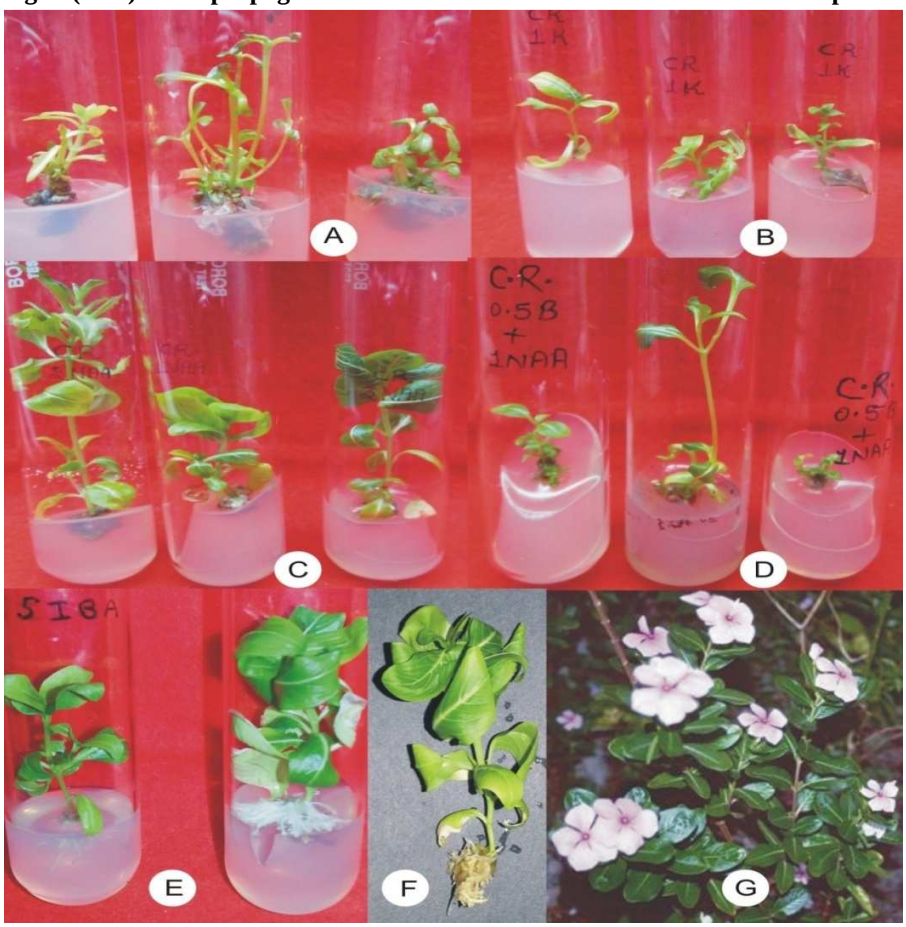
Table-4: Effect of auxin (IBA) on root induction from isolated shoot of *Catharanthus roseus*

Hormone Concentration (mg/ l)	Number of roots/explants	Root length (in cm)	Rooting Response (%)
1.0 IBA	1.08±0.19	0.51±0.05	73
2.0 IBA	1.38±0.37	0.92±0.10	78
3.0 IBA	1.40±0.52	1.06±0.08	80
4.0 IBA	2.80±0.73	0.43±0.33	85
5.0 IBA	3.60±0.51	1.68±0.32	90

Medium: MS+ additives; mean± SD, n= 7 replicates.

Means having the same letter in each Colum do not different significantly at P< 0.05 (Tukey's test)

Fig.1: (A-G) Micropropagation of *Catharanthus roseus* from nodal shoot explants



A. Shoot multiplication on MS medium supplemented with 1.0 mg/l BAP, **B.** Shoot multiplication on MS medium supplemented with 1.0 mg/l Kn, **C.** Shoot multiplication on MS medium supplemented with 2.0 mg/l NAA, **D.** Shoot multiplication on MS medium supplemented with 0.5 mg/l BAP+1.0 mg/l NAA, **E.** In vitro root induction on ¼ of MS medium supplemented with 5.0 mg/l IBA, **F.** 4 weeks old rooted plant for hardening, **G.** well growing plant in green house.

Fig.2: Effect of cytokine (BAP and Kn) on shoot proliferation from nodal shoot explants of *C. roseus*

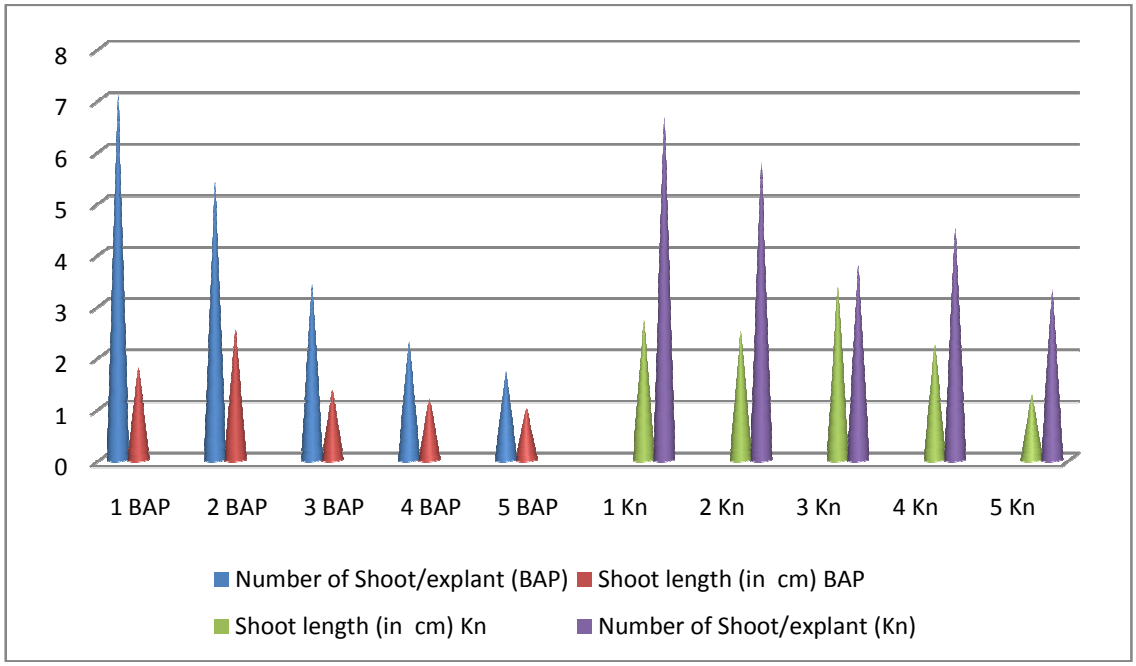


Fig.3: Interactive effect of cytokine (BAP + NAA) on shoot multiplication by subculture of shoot clumps of *C. roseus*

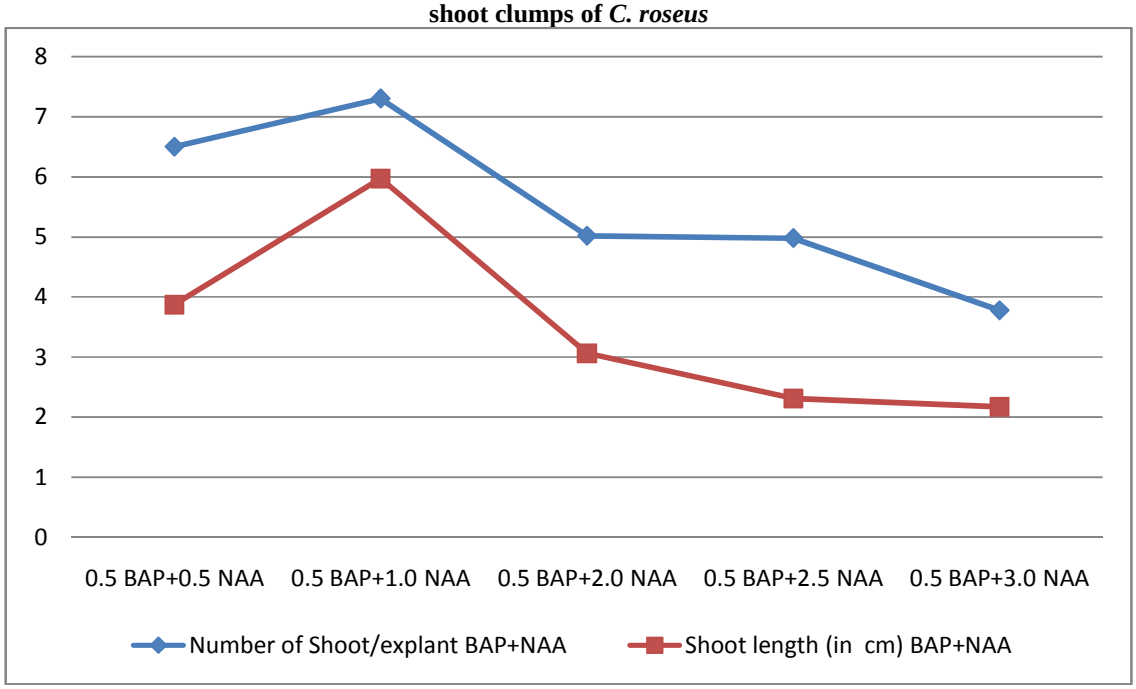


Fig.4: Effect of Auxin (NAA & IAA) on shoot multiplication by subculture of shoot clumps of *C. roseus*

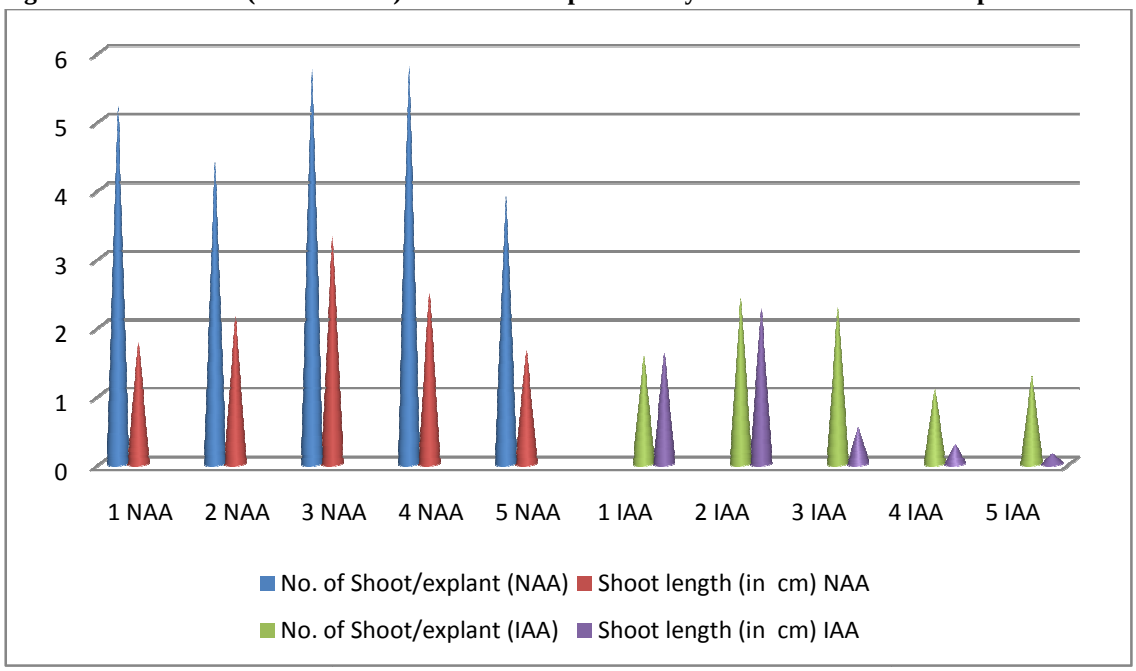
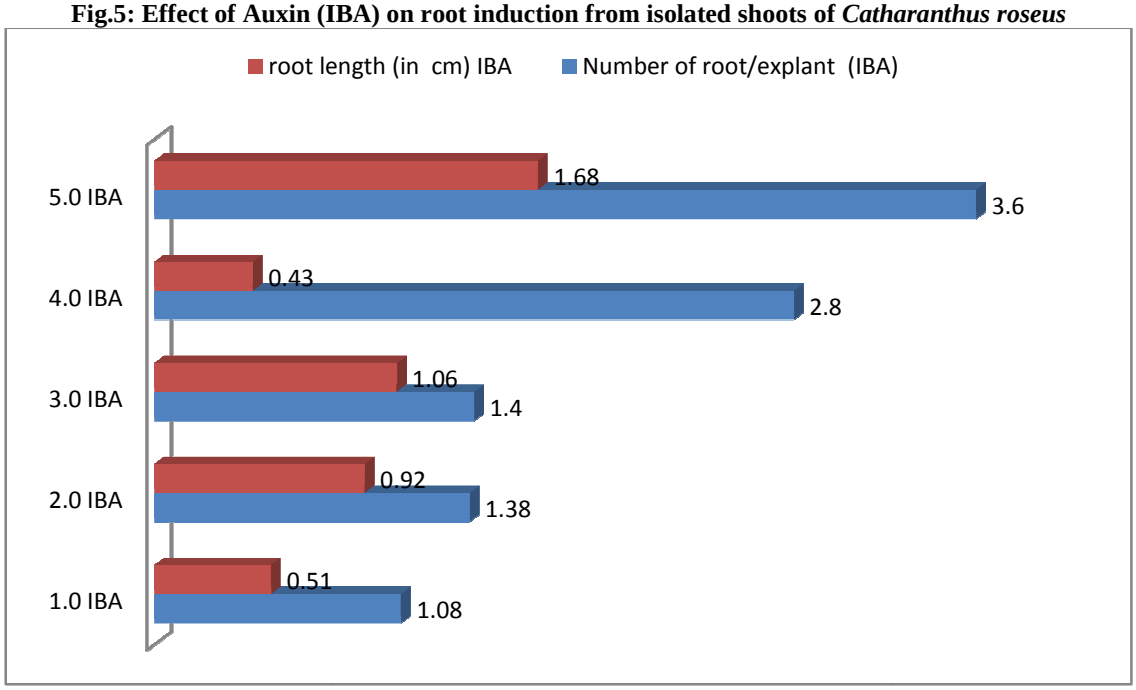


Fig.5: Effect of Auxin (IBA) on root induction from isolated shoots of *Catharanthus roseus*



RESULTS AND DISCUSSIONS FOR *Bacopa monnieri*

The nodal explants, when inoculated on MS medium containing BAP and Kn in the range 1.0-5.0 mg/l showed enhanced shoot proliferation. BAP at its 1.0 mg/l concentration evoked best response. Shoots after their initial proliferation on medium containing 1.0 mg/l BAP were sub-cultured on same fresh medium after every 21 days. Incorporation of BAP or Kn into MS medium supported multiplication of shoots in culture, BAP proved to be a better choice than Kn and the maximum number of shoots were obtained on its 1.0 mg/l concentration (Table 5, Fig.6 A, B & Fig.7). When BAP was used in combination with Kn a variety of responses were observed (Table 6, 7 & Fig. 6 C, D, Fig. 8). But best response was observed on medium containing 0.5 mg/l BAP + 2.0 mg/l Kn (Average number of shoots 3.42±0.39,

shoot length 7.54±0.31 cm) and in another combination of Kn and BAP, best response was observed on medium containing 0.5 mg/l Kn+1.0 mg/l BAP (Average number of shoots 4.98±0.74, shoot length 3.06±0.22 cm). The full or half strength of MS medium without any PGR was failed to induce rooting of regenerated shoots. However, shoots were capable to induce root when cultured on medium containing auxins.

Auxins in different concentration induced rooting when incorporated in the medium containing ¼ of MS salts. The best rooting response, however, was observed on medium containing 2.0mg/l IBA, where roots measuring 1.68±0.32 cm (average) were formed (Table 8, Fig.6 & Fig. 9). *In vitro* rooted plantlets were initially hardened in culture room conditions where leaves expanded. After 3 weeks, the plantlets were shifted to mist house. There was an increase in length of shoots and new leaves emerged which expanded quickly (Fig.6 G).

Leaf petiole explants were used for the purpose of callus induction. Highest diameter of callus was observed on MS Medium fortified with 0.25 mg/l 2,4-D+ 0.5 mg/l Kn (callus diameter 3.6 cm) and 0.25 mg/l 2,4-D+ 0.1mg/l BAP (callus diameter 3.2 cm), (Table 9, Fig.6 H-I, Fig. 10).

Table-5: Effect of Cytokinin (BAP and Kn) on shoot proliferation from Nodal shoot explant of *B. monnieri*

Hormone Con. (mg/ l)	Hormone Con. (mg/ l)	Response (%)	No. of Shoot/explant (mean±SD)	Shoot length (in cm) (mean±SD)
BAP	Kn			
1.0	-	80	3.42±0.58	6.51±0.76
2.0	-	70	2.28±0.71	6.56±0.84
3.0	-	65	2.71±0.56	7.62±0.53
4.0	-	55	3.28±0.36	5.08±0.51
5.0	-	40	2.85±0.51	3.31±0.33
-	1.0	55	2.28±0.36	6.47±0.29
-	2.0	75	2.42±0.39	6.30±0.26
-	3.0	60	1.85±0.27	6.15±0.24
-	4.0	40	1.57±0.40	5.70±0.41
-	5.0	30	1.28±0.36	4.92±0.51

Medium: MS+ additives; mean± SD, n= 7 replicates

Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table-6: **Interactive effect of Cytokinin (BAP+ Kn) on shoot multiplication by Subculture of shoot clumps of *B. monnieri***

Hormone Con. (mg/ l)	No. of Shoot/explant	Shoot length (in cm)	Shooting Response (%)
0.5 BAP + 0.5 Kn	1.71±0.38	3.70±0.28	70
0.5 BAP + 1.0 Kn	2.14±0.51	4.71±0.29	80
0.5 BAP + 2.0 Kn	3.42±0.39	7.54±0.31	90
0.5 BAP + 3.0 Kn	2.70±0.36	5.70±0.41	85
0.5 BAP + 4.0 Kn	2.57±0.40	6.70± 0.39	82

Medium: MS+ additives; mean± SD, n= 7 replicates

Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table-7: Interactive effect of Cytokinin (Kn+ BAP) on shoot multiplication by Subculture of shoot clumps of *Bacopa monnieri*

Hormone Con. (mg/ l)	No. of Shoot/explant	Shoot length (in cm)	Shooting Response (%)
0.5 Kn+0.5 BAP	2.26±0.24	3.70±0.28	70
0.5 Kn+1.0 BAP	4.98±0.74	3.06±0.22	95
0.5 Kn+2.0 BAP	2.31±0.48	3.87±0.39	80
0.5 Kn+3.0 BAP	3.78±0.57	3.06±0.22	85
0.5 Kn+4.0 BAP	2.26±0.24	2.60±0.51	83

Medium: MS+ additives; mean± SD, n= 7 replicates

Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table-8 : Effect of Auxin (IBA) on root induction from isolated shoot of *Bacopa monnieri*

Hormone Con. (mg/ l)	No. of roots/explants	Root length (in cm)	Rooting Response (%)
0.5 IBA	1.08±0.19	0.43±0.33	78
1.0 IBA	1.38±0.37	0.92±0.10	80
1.5 IBA	1.40±0.52	1.06±0.08	85
2.0 IBA	3.60±0.51	1.68±0.32	95
2.5 IBA	2.80±0.73	0.51±0.05	73

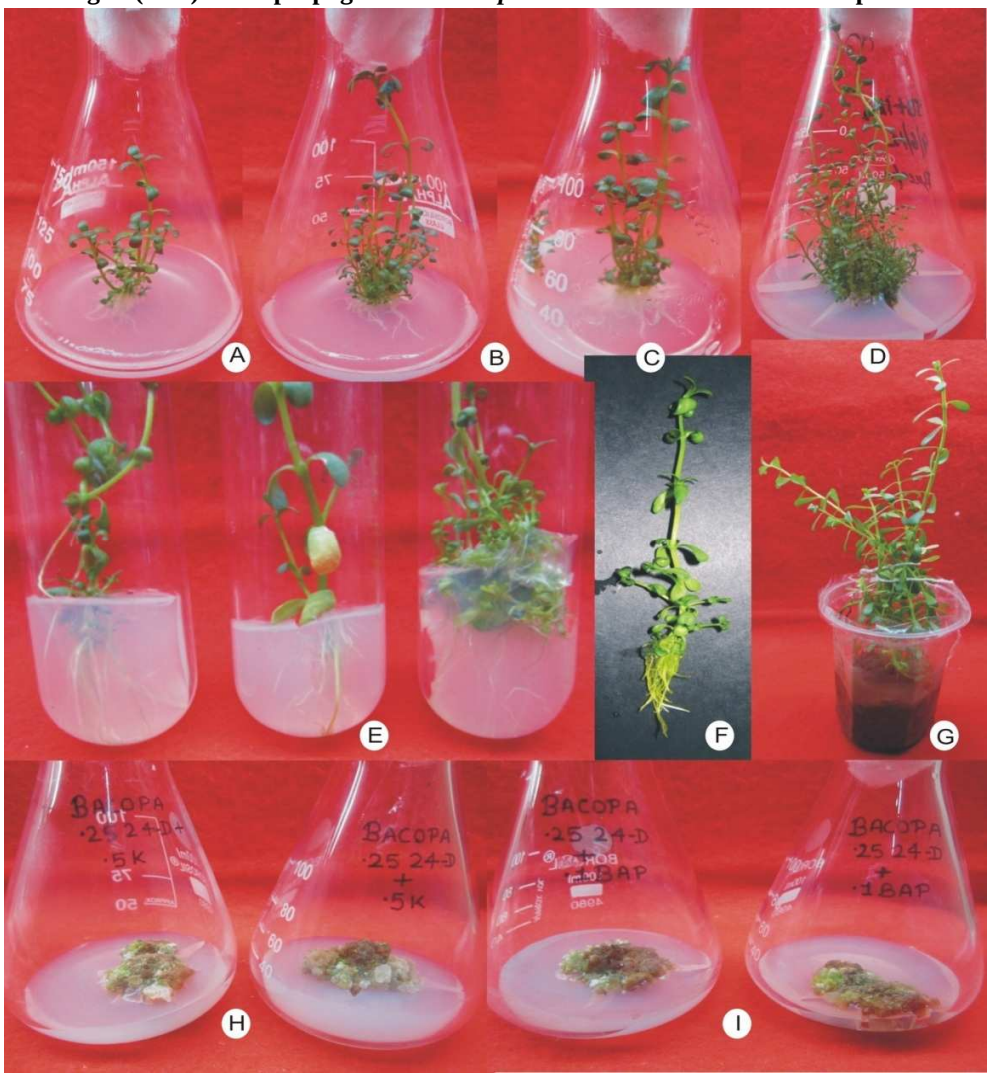
Medium: MS+ additives; mean± SD, n= 7 replicates
Means having the same letter in each Colum, do not different significantly at P< 0.05 (Tukey's test)

Table-9: Effect of different Hormones on Callus proliferation and Morphology

Hormone Conc. (mg/l)	Callus diameter after 7 weeks subculture (cm)	Callus proliferation Scoring	Color of callus	Morphology of callus
2,4-D + Kn				
0.25 + 0.3	1.9	++	Brownish	Friable
0.25 + 0.4	2.4	+++	Whitish brown	Nodular
0.25 + 0.5	3.6	++++	Brownish green	Compact
2,4-D + BAP				
0.25 + 0.1	3.2	++++	Dark Brownish green	Trodden
0.25 + 0.2	2.4	++	Whitish green	Friable Nodular
0.25 + 0.3	2.6	+++	Brownish green	

'++++' Intense
'+++' Moderate
'++' Meager

Fig.6: (A-G) Micropropagation of *Bacopa monnieri* from nodal shoot explants



A. Shoot multiplication on MS medium supplemented with 1.0 mg/l BAP, **B.** Shoot multiplication on MS medium supplemented with 2.0 mg/l Kn, **C.** Shoot multiplication on MS medium supplemented with 0.5 mg/l BAP+2.0 mg/l Kn, **D.** Shoot multiplication on MS medium supplemented with 0.5 mg/l Kn+1.0 mg/l BAP, **E.** In vitro root induction on ¼ of MS medium supplemented with 2.0 mg/l IBA, **F.** 4 weeks old rooted plant for hardening, **G.** hardened plant growing on soilrite moistened with basal medium, **H**. Callus induction from leaf petiole on MS medium supplemented with 0.25 mg/l 2,4-D +0.5 mg/l Kn, **I.** Callus induction from leaf petiole on MS medium supplemented with 0.25 mg/l 2,4-D +0.1 mg/l BAP.

Fig.7:**Effect of cytokine (BAP and Kn) on shoot proliferation from nodal shoot explants of *B. monnieri***

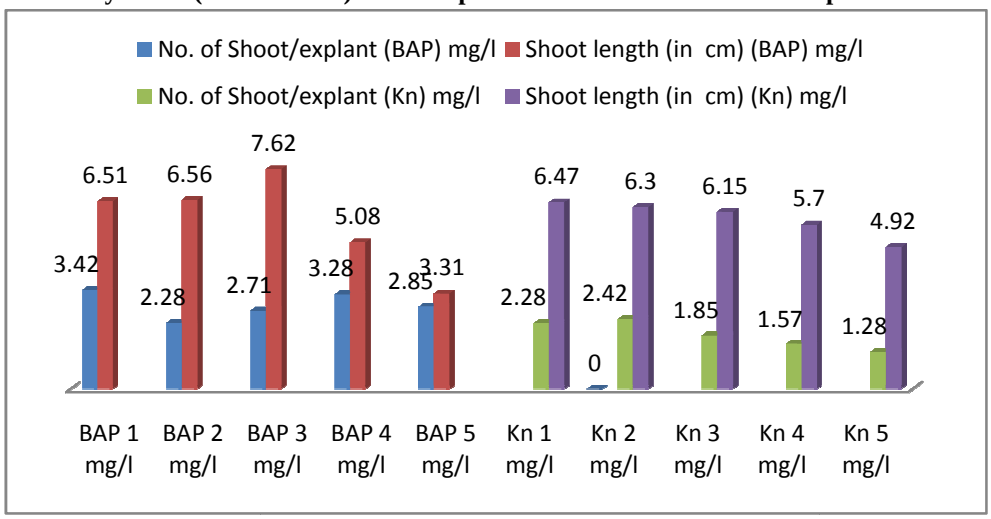


Fig.8:Interactive effect of cytokine (BAP + Kn) on shoot multiplication by subculture of shoot clumps of *B. monnieri*

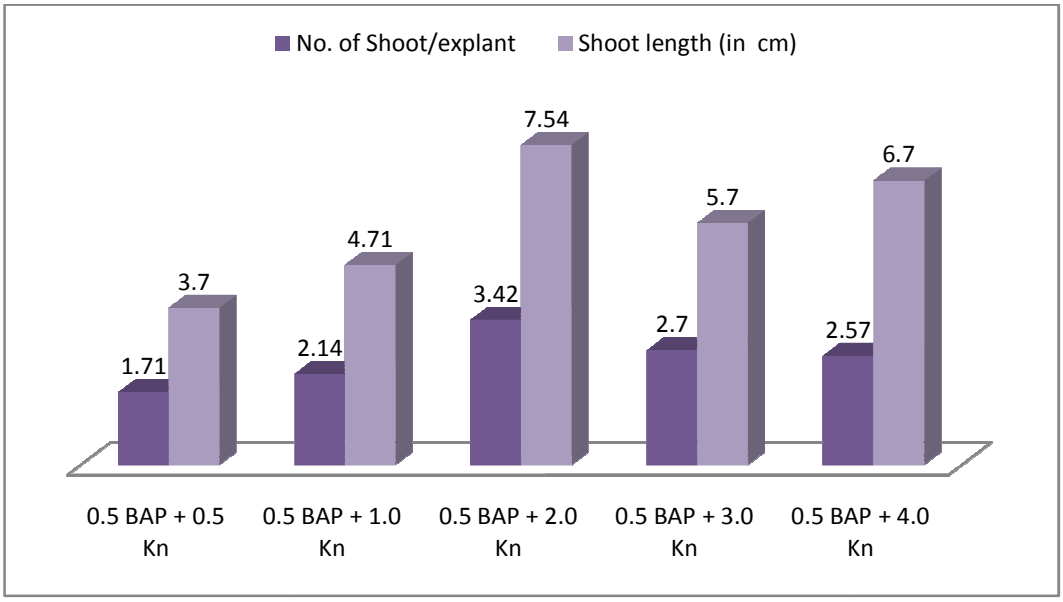


Fig.9: Interactive effect of cytokine (Kn + BAP) on shoot multiplication by subculture of shoot clumps of *Bacopa monnieri*

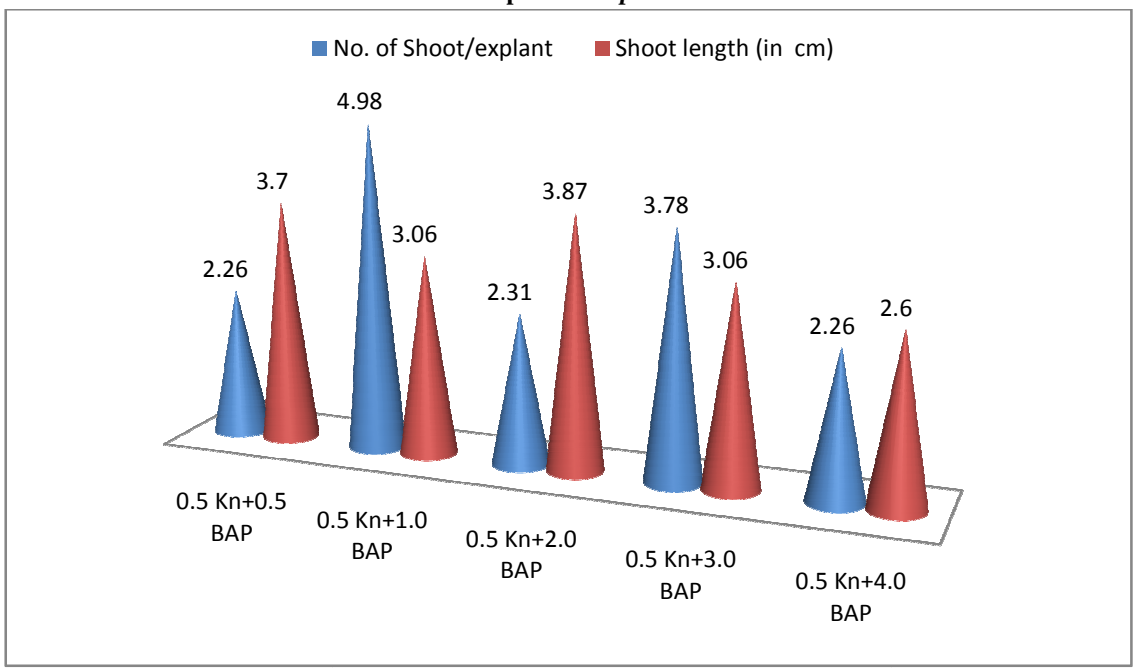
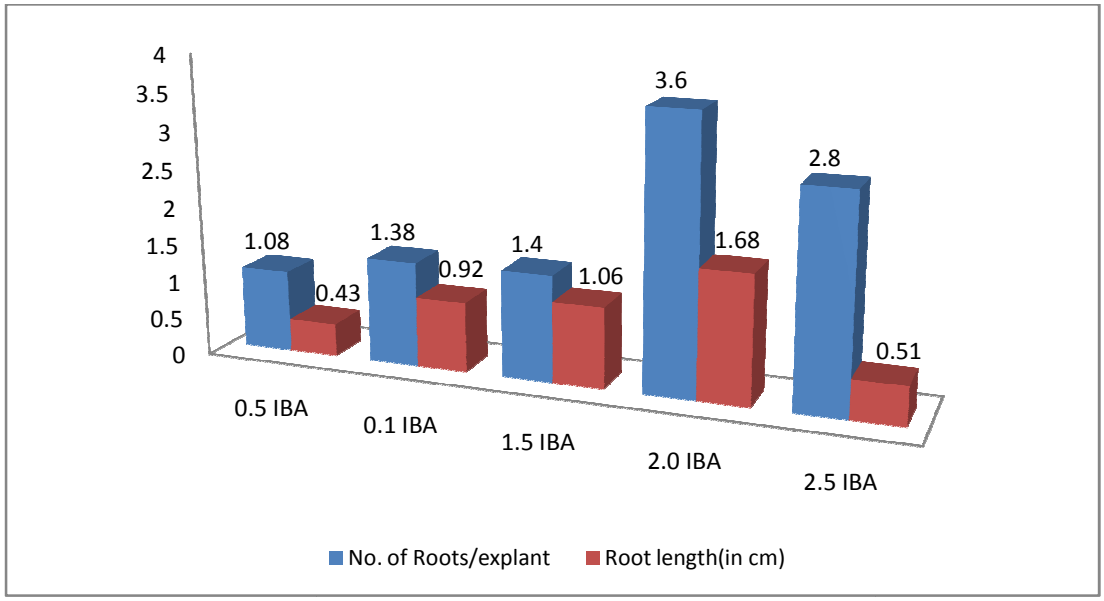


Fig.10: Effect of Auxin (IBA) on root induction from isolated shoots of *Bacopa monnieri*



CONCLUSION

The seedling derived explants, being juvenile, are frequently used for micropropagation, as they are easy to establish in culture. In *Catharanthus roseus*, MS medium containing 1.0 mg/l BAP was best for culture initiation. We have found that *Catharanthus roseus* culture grew better on MS medium in comparison to other media. In *Catharanthus roseus* 1.0 mg/l BAP was most suitable for shoot multiplication. We also observed improvement in shoot multiplication by different concentrations of NAA (0.5-3.0 mg/l) in medium along with BAP (0.5 mg/l). Best shooting response was observed on media containing 0.5 mg/l BAP+ 1.0 mg/l NAA (Average number of shoots 7.30±0.64) and 0.5 mg/l BAP+ 1.0 mg/l NAA (Average shoot length 5.97±0.17cm).

IBA (Auxin) has been widely used as root induction hormone under *in vitro* and *in vivo* condition. We also found positive role of IBA during *in vitro* rooting. In *Catharanthus roseus*, 5.0 mg/l IBA proved to be the best for in vitro rooting. The *in vitro* rooted plants were hardened first under controlled conditions of culture room and then shifted to mist house where they exhibited and hence, growth and 90% survival.

The seedlings derived from explants, being juvenile, are frequently used for micropropagation, as they are easy to establish in culture. In *Bacopa monnieri*, MS medium containing 1.0 mg/l BAP was the best for culture initiation. We have found that *Bacopa monnieri* culture grew better on MS medium in comparison to other media. In *Bacopa monnieri* 1.0 mg/l BAP was most suitable for shoot multiplication. We also observed improvement in shoot multiplication by different concentrations of Kn. (0.5-4.0 mg/l) in medium along with BAP (0.5 mg/l). Best shooting response was observed on media containing 0.5 mg/l BAP+ 2.0 mg/l Kn (Average number of shoots 3.42±0.39, Average shoot length 7.54±0.31 cm). In different concentrations of Kn. (1.0-5.0 mg/l) *Bacopa monnieri* give best shooting response in 2.0 mg/l Kn. We also observed improvement in shoot multiplication by different concentrations of BAP (0.5-4.0 mg/l) in medium along with Kn. (0.5 mg/l). Best shooting response was observed on media containing 0.5 mg/l KN+1.0 mg/l BAP (Average number of shoots 4.98±0.74, shoot length 3.06±0.22 cm).

IBA (Auxin) has been widely used as root induction hormone under *in vitro* and *in vivo* condition. We also found positive role of IBA during *in vitro* rooting. In *Bacopa monnieri*, 2.0mg/l IBA proved to be best for *in vitro* rooting. The *in vitro* rooted plants were hardened first under controlled conditions of culture room and then shifted to misthouse where they exhibited growth with 90% survival. Most responsive callus induction was observed on MS medium supplemented with 0.25 mg/l 2,4-D +0.5 mg/l Kn and 0.25 mg/l 2,4-D +0.1 mg/l BAP.

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