GAVIN PUBLISHERS

Advances in Proteomics and Bioinformatics

Research Article

Zafar NUA, et al. Adv Proteomics Bioinform: APBI-104.
DOI: 10.29011/APBI -104. 100004

Efficient Protocol on Callus Induction of *Cassia angustifolia* for Remarkable Production

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Citation: Zafar NUA, Raja NI, Javed U, Yasmeen F (2018) Efficient Protocol on Callus Induction of Cassia angustifolia for Remarkable Production. Adv Proteomics Bioinform: APBI-104. DOI: 10.29011/APBI -104. 100004

Received Date: 06 December, 2017; **Accepted Date:** 02 January, 2018; **Published Date:** 07 January, 2018

Abstract

Cassia angustifolia Vahl *(C. angustifolia)*, commonly known as senna, is a medicinally valuable drought resistant shrub of family Leguminosae. For mass production, rapid propagation and conservation, callus induction and regeneration efficiency in seeds of Cassia is analyzed. Various concentrations of both 2,4-D and KN in MS media were used. The higher concentrations of both hormones were more effective for callus induction. In limited space and time, 2,4-D and KN found to be best for callus induction. For shoot proliferation, BAP and Kinetin showed the suitability. While, IBA and NAA were appropriate for rooting of proliferated shoots. In this way, an efficient protocol for the micro propagation of *C. angustifolia* has been standardized through callus induction, shoot regeneration and proliferation.

Introduction

C. angustifolia Vahl, commonly known as senna, is a medicinally valuable drought resistant shrub of family Leguminosae. It is nativeto Saudi Arabia and has been recommended for developing wastelands as Leguminosae *s*pecies are known for their recalcitrant nature. Some successful attempts have been made on *in vitro* organogenesis of *C. fistula, C. siamea* [1] and *C. alata* [2]. Incidentally, there is no report on *in vitro* regeneration of *C. angustifolia* on seedling derived explants. Reports on its cultivation and genetic improvement are also limited [3,4]. Regeneration via calli can be the potent source of producing soma clonal variants in plants and thus the improvement of the species.

C. angustifolia was initially discovered growing wild in and around the prehistoric and sacred city of Makkah, in the heart of the old province Hijaz. The plant develops in plenty and was first used as herbal medicine by the Holy Prophet Muhammad (Peace Be Upon Him) [5]. At herbal shops in India, Pakistan and Arabian countries, it is traded under the name of Senna or Sana makkahi, and is considered to be a cure as a cleanser of the digestive system and stimulant for the whole body. The Holy Prophet Muhammad (Peace Be Upon Him) said; "If there is any cure against death, it is Senna, the delighted, the charming one" [6]. Now a day, Senna is scattered globally, particularly in Pakistan, India, Arabian countries, Sudan, China, Kenya, Europe, Britain, etc. Senna is commonly used in conventional medicine of China, Indo-Pakistan, Africa, and is also engaged in Western Allopathic System of medicine [7]. *C. angustifolia* is regularly used for digestive disorders, constipation, stimulant, depression, asthma, eczema and other skin diseases.

The highest percentage of callus induction and shoots proliferation was reported *in vitro* plant regeneration and flowering from young leaf explants of chicory (*Cichorium intybus* L. cv. Focus) in vermiculite and later shifted to the field [8]. An improved protocol for multiple shoot regeneration from nodal segments of wood apple *Aegle marmelos* L, a medicinal tree, cultured on Murashige and Skoog [9] (MS) medium supplemented with various concentrations of auxins and cytokinins was presented. The protocol provides a basis for germplasm conservation and for further investigation of bioactive constituents of the plant [10].Callus induction and plant regeneration with alkaloids accumulation in stem and shoots tip explants of *Phyla nodiflora* were acclimatized and established in soil with 90% survival rate which could be effective method for the conservation and clonal propagation [5].There is a need to analyze the different concentrations of plant growth regulators added in a suitable basal medium for best callus induction.

In vitro callus induction and regeneration is an advantageous practice for mass production, rapid propagation and conservation for medicinal plants. *In vitro* propagation of *Cardiospermum*

helicacabum from leaf and nodal explant derived calli cultured on MS medium and highest number of adventitious shoots (28 per callus) formed through which roots developed within 45 days [11]. For micro propagation of *C.* angustifolia Vahl, from root explants from 30-days-old aseptic seedlings were cultured on MS medium supplemented with different plant growth regulators with survival rate [12]. Present work was done to establish efficient method for callus induction and regeneration efficiency in seeds of Cassia by application of both 2,4-D and KN in MS media.

Materials and Methods

Collection of Seeds

Mature and healthy seeds were collected from pods of *C.* angustifolia Vahl, bought from different grocery markets in Pakistan. Seeds were also purchased and imported from Saudi Arabia using authenticated means.

Seed Sterilization

Seed sterilization process was conducted in laminar flow chamber to maintain maximum sterile conditions following the procedure of Rashid et al., [13]. Healthy seeds were washed with autoclaved distilled water first and then soaked in the 70% ethanol for one-minute leading to subsequent washing with autoclaved distilled water. After this, the seeds were further sterilized by using suitable commercial bleach such as 5% Clorox for 20 minutes followed by washing thrice with autoclaved distilled water. These sterilized seeds were placed in sterilized petridishes having filter papers.

Media Preparation and Seed Inoculation

For callus induction, MS medium, salts and vitamins, 3% (w/v) sucrose and 0.8% (w/v) Gelrit e® supplemented with different concentrations of 2, 4-dichlorophenoxy acetic acid (2, 4-D) were used. The media was adjusted to pH 5.75 and autoclaved for 15 min., at 121°C through standard procedure. Different PGHs and their concentration combinations used for callus induction, shoot proliferation and rooting (Table 1). Sterilized seeds were inoculated on the gel solidified, autoclaved MS media supplemented with 2, 4-D, in glass tubes (18 mm in diameter and 150 mm in depth). 8 ± 1 ml medium was taken in each culture vessel and one seed was planted per culture vessel carefully under sterilized conditions.

Treatments	Concentration Combinations (mg/L)
T_o	0.0+ 0.0
T_1	0.0+1.0
T_2	0.0+3.0
T_3	0.0+5.0
T_4	0.0+7.0
T_5	1.0+0.0
T_6	1.0+1.0
T_7	1.0+ 3.0
T_8	1.0+5.0
T_9	1.0+7.0
T_{10}	3.0+0.0
T_{11}	3.0+1.0
T_{12}	3.0+3.0
T_{13}	3.0+5.0
T_{14}	3.0+7.0
T_{15}	5.0+0.0
T_{16}	5.0+1.0
T_{17}	5.0+3.0
T_{18}	5.0+5.0
T_{19}	5.0+7.0
T_{20}	7.0+0.0
T_{21}	7.0+1.0
T_{22}	7.0+3.0
T_{23}	7.0+5.0
T_{24}	7.0+7.0

Table 1: Different PGHs and their concentration combinations used for Callus Induction, Shoot Proliferation and Rooting.

- **Combinations of PGHs used**

1) Callus induction:

o IBA+KN

2) Shoot Proliferation:

o BAP+IBA

o KN+BAP

3) Rooting:

o IBA+NAA

o IBA+IAA

Maintenance of Callus Cultures

Cultures were then transferred and maintained in environmentally controlled room under continuous illumination of 1500 lux emitted by general electric fluorescent tubes. Temperature was maintained at 25 ± 3°C throughout the growth period for optimum growth and to control contamination of the cultures. About 4-5 weeks were permitted for adequate induction

and growth of the calli. At the end of each culture passage, non-embryogenic calli, were recognized on the basis of visual estimates by naked eye. Non-embryogenic calli were dissected away from the embryogenic callus and discarded.

Regeneration

The plant regeneration ability of *C. angustifolia* was assessed using MS salts and vitamins, 3% (w/v) sucrose and 0.2% (w/v) Gelrite®. Different combinations (0, 1.0, 3.0, 5.0 and 7.0) of growth regulators, IBA, 6-BAP, KIN, IAA and NAA at the rate of 1-5 mg/l were assayed to induce shoot and root differentiation and subsequent regeneration of plants from different aged calli. Calli bearing green spots and the total number of regenerates were counted after a time interval of 6-8 weeks. Each developing green shoot with an initiated root system was counted as one plant. The established plantlets were subjected to hardening and acclimatization by transferring to sterile soil and then to the field.

Acclimatization

In vitro explants of *C. angustifolia* were removed from the rooting media. The rooted shoots were then washed in distilled water to get rid of any basal callus. The plantlets were then shifted to plastic pots containing autoclaved garden soil and covered with polythene bags to sustain the relative humidity. These pots were placed under shade and checked regularly for light and temperature conditions. Polythene bags were opened after about two weeks in order to acclimatize plants to field conditions and to observe growth parameters.

Statistical Analysis

All experiments were conducted in factorial experimental arrays of treatments based on completely randomized design. All treatments were simulated with a minimum of ten replicates per treatment and repeated three times. Data was analyzed statistically through two-way ANOVA using Microsoft Excel 2007 software [14]. The calculation of means was carried out by LSD and represented as mean ± S.E

Results and Discussion

Effect of Varying Concentrations of 2, 4-D and KN on Callus Induction Percentage

For analyzing the effects of 2,4-D and JN on callus induction percentage, various concentration of both hormones was used. There is a significant difference between treatments concerning callus induction percentage in *C. angustifolia* Vahl at $p<0.05$. After about 4th week of inoculation, callus induction was started in seeds (Figure 1a). Among different concentrations of auxins (2,4-D) and cytokinins (KN) callus induction percentages were noteable from 0 to 100 %(Table 2). The most effective treatment was $T_{17}$with 93.43 % (Figure 1b). The minimum callus induction was revealed in T5. After 7th week and 2nd subculture of callus induction medium shoot initiation and proliferation got started (Figure 1c). KN are naturally stirring plant hormones that uphold cell division and are crucial for regular plant growth and expansion [15]. High concentrations of 2, 4-D and KN was found more effective for *In* vitro callus induction in *Convolvulus alsinoides* [16,17].

Treatment (2,4-D+KN)	Mean Callus Induction (%)
T_0	0
T_1	53.23
T_2	41.76
T_3	46.56
T_4	58.43
T_5	33.23
T_6	63.43
T_7	53.23
T_8	60.14
T_9	43.43
T_{10}	55.68
T_{11}	66.56
T_{12}	63.43
T_{13}	36.76
T_{14}	56.56
T_{15}	81.56
T_{16}	73.53
T_{17}	93.43
T_{18}	66.56
T_{19}	87.48
T_{20}	76.56
T_{21}	86.76
T_{22}	76.76
T_{23}	46.86
T_{24}	63.23
LSD5%	9.97%
No significant difference between any two means sharing a letter at $p<0.05$	

Table 2: Influence of 2, 4-D and KN on Callus Induction Percentage.

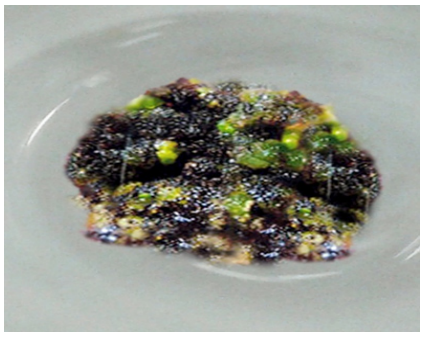


Figure 1a: Callus induction after 4th week of inoculation.

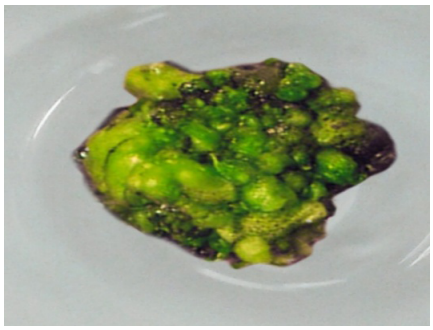


Figure 1b: 5.0 mg/L 2, 4-D and 3.0 mg/L KN with 93.43 % callus induction.

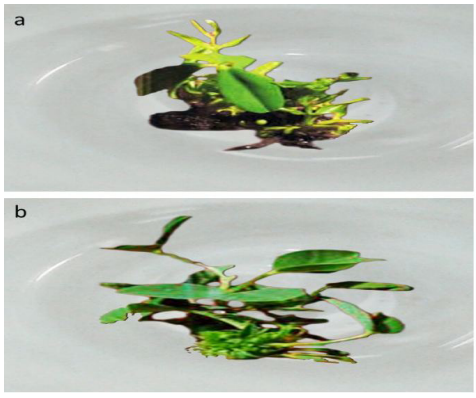
Figure 1c:5.0 mg/L 2, 4-D and 7.0 mg/L KN with 87.48% callus induction.

Effect of Different Concentrations of (BAP+ IBA) and (KN + BAP) on Shoot Number

The effect of different combinations of growth hormones on shoot number, various concentrations and combinations of BAP, IBA, and KN were used (Table 3). BAP free media (T_0) showed no adventitious shoot in the apical and axillary buds (Figure 2a). Average number of shoots (7.59) was shown by T_{17} (Figure 2b).

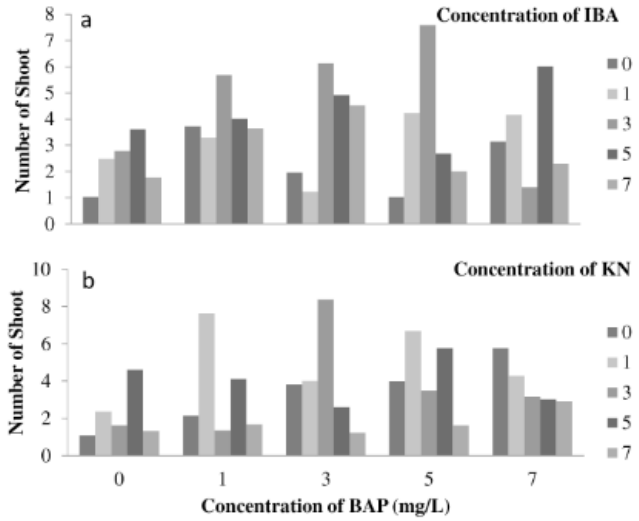
Treatment (BAP+IBA)	Mean Shoot Number	Mean Shoot Length(cm)
T_0	1.03	1.30
T_1	2.49	3.49
T_2	2.78	1.38
T_3	3.61	1.52
T_4	1.76	1.24
T_5	3.72	1.92
T_6	3.30	4.23
T_7	5.69	2.47
T_8	4.01	2.51
T_9	3.65	1.88
T_{10}	1.96	4.20
T_{11}	1.23	2.36
T_{12}	6.14	1.25
T_{13}	4.92	2.12
T_{14}	4.53	1.02
T_{15}	1.02	3.24
T_{16}	4.23	5.01
T_{17}	7.59	5.06
T_{18}	2.68	3.27
T_{19}	2.00	3.46
T_{20}	3.14	4.00
T_{21}	4.16	2.69
T_{22}	1.39	4.83
T_{23}	6.02	3.36
T_{24}	2.30	2.99

Table 3: Effect of Different Concentration of BAP and IBA on Shoot Number and Shoot Length of *Cassia angustifolia* Vahl.



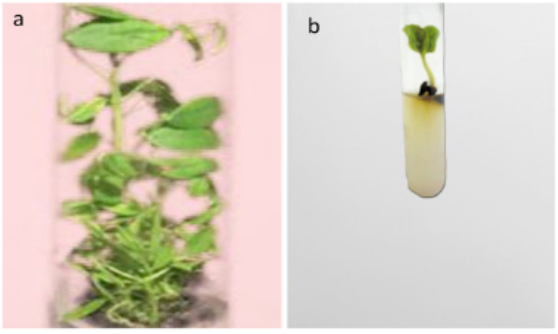
Figures 2(a-b): Initiation of shoot proliferation at 5.0 mg/L BAP and 3.0 mg/L IBA and improved shoot number at 5.0 mg/L BAP and 3.0 mg/L IBA.

The minimum shoot number of 1.02was obtained at T_{15}.Shoot number was significantly increased at 5 mg/l BAP amended with 3 mg/l IBA (Figure 3a). Relations of KN and BAP were incredibly considerable for shoot numbers (Figure 3b) as maximum shoot number was achieved at 3 mg. l BAP amended with 3 mg/l KN followed by 1 mg/l BAP amended with 1 mg/l KN (Figure 3b). Thomas et al. [18] tested different 2,4-D and KN for regeneration of ash gourd and found highest number of shoots per culture on MS medium equipped with BAP. However, at high concentration of BAP as both apical and axillary explants gave dense clumps of new shoots with a lot of axillary buds but showed no shoot elongation [19].



Figures 3(a-b): Interaction of IBA with BAP and KN at different concentrations for Shoot Number.

The maximum number of shoots (8.36) was obtained at T_{12} (Figure 4a) when MS media enhanced with 3.0 mg/L KN and 3.0 mg/L BAP. This was followed by $T_6$with 7.64 average shoot number (Table 4). The minimum number of shoots (1.09) was attained at $T_0$when no PGHs were added. Maximum shoot initiation (80%) was accomplished with BAP 3.0 mg/L + Kinetin 3.0 mg/L. [20] stated that maximum shoot initiation (80%) was accomplished with BAP 3.0 mg/L + Kinetin 3.0 mg/L in rose. These results are exactly similar to our conclusions for shoot proliferation in *C. angustifolia.* The addition of 3.0mg/L of BAP can be used as an appropriate component for the outstanding growth of *Tectona grandis* (L.) tissue culturing [21].



Figures 4(a-b): Interaction of IBA and BAP at different concentrations for Shoot Length.

Treatment (KN+BAP)	Mean Shoot Number	Mean Shoot Length (cm)
T_0	1.09	2.53
T_1	2.37	1.62
T_2	1.62	1.40
T_3	4.61	1.73
T_4	1.32	1.26
T_5	2.14	3.44
T_6	7.64	3.12
T_7	1.35	2.60
T_8	4.11	4.02
T_9	1.68	3.38
T_{10}	3.82	2.57
T_{11}	4.00	3.37
T_{12}	8.36	1.61
T_{13}	2.60	3.28
T_{14}	1.23	3.43
T_{15}	3.99	4.24
T_{16}	6.69	5.63
T_{17}	3.49	2.29
T_{18}	5.76	3.48
T_{19}	1.62	2.75
T_{20}	5.76	3.99
T_{21}	4.28	4.30
T_{22}	3.18	5.97
T_{23}	3.02	1.02
T_{24}	2.90	2.09

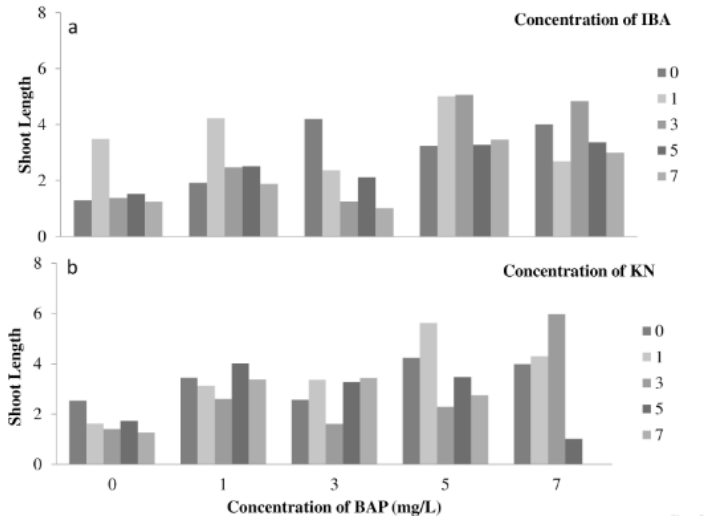
Table 4: Effect of Different Concentration of KN and BAP on Shoot Number and Shoot Length of *Cassia* angustifolia Vahl.

Effect of Different Concentration of (BAP + IBA) and (KN + BAP) on Shoot Length

Average shoots length of (1.30 cm) at T_0 (Figure 5a) where no PGHs were added to the media. When different combinations of BAP and IBA were used in MS media, the maximum shoot length (5.06 cm) achieved (Figure 5b) at T_{17}. The minimum shoot length (1.02 cm) was obtained at T_{14} (Table 3). PGHs have a great impact on shoot growth both in terms of number and length. High concentration of BAP and low concentration of IBA are more beneficial for shoot proliferation in *Cassia*.

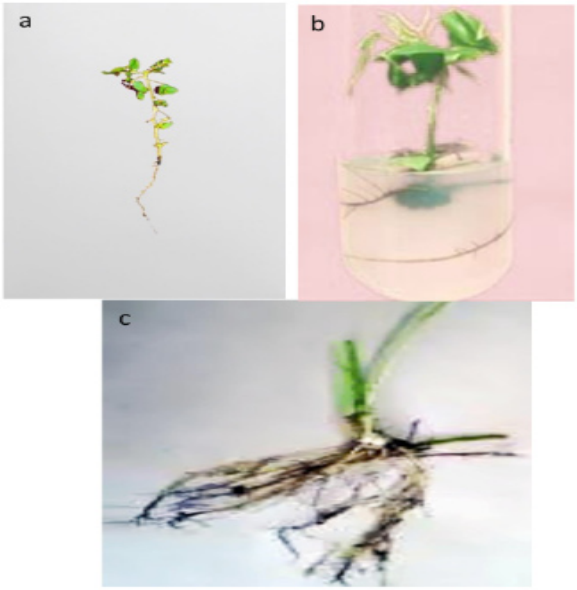
Figures 5(a-b): Interaction of KN and BAP at different concentrations for Shoot Number.

Varying concentration of BAP and IBA in culture media was reflected to shoot length at $p< 0.05$ (Figure 6). Among various BAP treatments, higher concentrations had a promotory effect on mean shoot length in both apical and axillary shoot buds of Avocado [22]. Rouzban et al., [23] supported our outcomes that higher application of cytokinins led to endorse shoot regeneration in Asian pear. Kumar *et al*. [24] reported that best culture was attained when MS media contain a combination of BAP and IBA in *Jatropa curcas*.



Figures 6(a-b): Effect of BAP in combination with IBA and KN on shoot length.

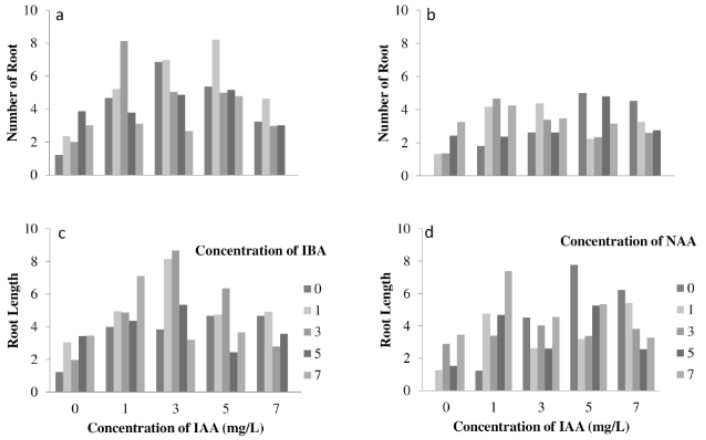
A shoot length of 2.53 cm (Table 4) was also achieved (Figure 7a) at T_0. The maximum average length of shoot (5.97 cm) was obtained at T_{22} (Figure 7b) which was followed by with 5.63 cm of average shoot length. The minimum shoot length (1.02 cm) was attained at T_{23}.Results showed that interaction of these two plant hormones was highly significant (Figure 6b). Gangopadhyay et al., [25] also reported the surprising growth assets with KN treatments and improved growth properties with BAP treatments. Media complemented with BAP and Kinetin had an influencing effect on shoot proliferation producing 2.5 and 2.4 shoots per inoculated shoot [26].



Figures 7(a-b): Interaction of KN and BAP at different concentrations for shoot length.

Effect of Different Concentrations of (IBA + IAA) and (IBA + NAA) on Number and Length of Root

Maximum number of roots (8.22) was found (Figure 7c) at T_{16} and number of roots (8.12) at T_7(Table 5).The minimum average root number obtained when no hormones were added at T_0. Combination of IBA and IAA at different concentrations was highly significant at $p<0.05$ (Figure 8a). The maximum response for root number (5.00) was shown by T_{15} (Table 6) which was followed by T_{18} having an average root number of 4.80 (Figure 8a). Average root number of 1.23 was found at T_1 supplemented with 1.0 mg/L of NAA (Figure 8b). The work was in agreement with Khosh and Sink [27] who reported NAA plus IBA augmented rooting better than IBA or NAA alone in *Rosa hybrida.* Tsipouridis et al. [28] revealed that auxins (IBA and NAA) participate in stimulation of roots induction to a greater extent. However, the optimum concentrations of auxins are recognized to be engaged in cell enlargement and are thought to be the controlling factor in rooting process.



Figures 8(a-d): Effect of IAA in combination with IBA and NAA number and length of root.

Treatment (IBA+IAA)	Mean Root Number	Mean Root Length (cm)
T_0	1.22	1.23
T_1	2.36	3.05
T_2	2.00	1.96
T_3	3.87	3.42
T_4	3.02	3.46
T_5	4.68	3.99
T_6	5.22	4.95
T_7	8.12	4.87
T_8	3.79	4.36
T_9	3.12	7.10
T_{10}	6.86	3.84
T_{11}	6.99	8.14
T_{12}	5.04	8.66
T_{13}	4.87	5.35
T_{14}	2.66	3.20
T_{15}	5.36	4.68
T_{16}	8.22	4.74
T_{17}	5.00	6.36
T_{18}	5.17	2.44
T_{19}	4.79	3.66

T_{20}	3.25	4.67
T_{21}	4.62	4.93
T_{22}	2.99	2.79
T_{23}	3.02	3.56
T_{24}	2.44	2.49

Table 5: Effect of Different Concentration of IBA and IAA on Root Number and Root Length of *Cassia angustifolia* Vahl.

Treatment (IBA+NAA)	Mean Root Number	Mean Root Length (cm)
T_0	0.00	0.00
T_1	1.32	1.26
T_2	1.36	2.89
T_3	2.43	1.52
T_4	3.26	3.46
T_5	1.80	1.23
T_6	4.18	4.76
T_7	4.66	3.39
T_8	2.37	4.68
T_9	4.24	7.40
T_{10}	2.63	4.52
T_{11}	4.37	2.62
T_{12}	3.39	4.02
T_{13}	2.62	2.60
T_{14}	3.47	4.55
T_{15}	5.00	7.78
T_{16}	2.24	3.20
T_{17}	2.33	3.38
T_{18}	4.80	5.26
T_{19}	3.16	5.34
T_{20}	4.52	6.22
T_{21}	3.26	5.43
T_{22}	2.60	3.82
T_{23}	2.76	2.56
T_{24}	2.14	3.27

Table 6: Effect of Different Concentration of IBA and NAA on Root Number and Root Length of *Cassia angustifolia* Vahl.

The maximum root length (8.66 cm) was obtained at T_{12} (Table 5) which was followed by average root length of 8.14 cm at T_{11} (Figure 8c). The minimum root length was observed at T_0 which was medium without any growth regulators (Figure 8d). The maximum average root length (7.78 cm) observed at (Figure 9) T_{15}.The next average root length was 7.40 cm at T_9 (Table 6). Wada et al, [29] revealed that root length was promoted by IBA as it enhances the synthesis of enzymes involved in enlargement of cells. Muller [30] stated that better uptake, transport, metabolism and successive gene activation might be the factors for the superior effects of IBA on root elongation in comparison to NAA. It was monitored that the excellence of the shoot and the root system at the end of the rooting period robustly manipulated by the type and concentration of auxins.

Figure 9: Interaction of IBA and NAA at different concentrations for root length.

Conclusion

It is concluded from this study that traditional methods of propagation can be altered by successful tool of micro propagation. It allows a rapid and efficient propagation of *C. angustifolia* Vahl in limited space and time. 2, 4-D and KN found to be best for callus induction. For shoot proliferation, BAP and Kinetin showed the suitability. While, IBA and NAA were appropriate for rooting of proliferated shoots. In this way, an efficient protocol for the micro propagation of *C. angustifolia* has been standardized through callus induction, shoot regeneration and proliferation.

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