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In vitro plant production through stem derived callus of senna (*Cassia angustifolia*)–a potential source of sennoside

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Abstract
An effective protocol was established for micropropagation of senna using callus developed from the stem. Senna is grown in different parts of India and is extensively used for its medicinal value. The nodal explants were taken from the twigs of the plant which were growing in the field. It was observed that when inoculated on MS + 5 µM N^6 -benzyladenine (BA) medium induced brownish black basal callus. Such calluses on transfer to Murashige and Skoog's medium containing BA or 2iP or Kn differentiated shoot buds within 7 to 10 days which organised shoots in next 10 days. Among these cytokinins, the best response was achieved on MS medium containing 5 µM BA, 100% cultures showed response (5.39±0.3 4 shoots per culture). Nearly 70 % excised shoots organised roots (3.25±0.45 roots per culture) on ½ strength Murashige and Skoog's medium containing 10µM NAA. Plantlets were gradually hardened in soilrite and acclimatised to soil. The protocol shall be useful for mass propagation as well as improvement of this crop.

Keywords: N^6- benzyladenine, *Cassia angustifolia*, murashige and skoog's medium, callus

Introduction
Cassia angustifolia Vahl belongs to the family Leguminosae and it is largely cultivated as cash crop (Anonymous, 1992) [1]. It's a medicinal plant, known as senna and rich source of sennosides which are anti-cancerous compounds (Chetri *et al*., 2014.) [2]. the anthraquinone glycosides (sennosides) which are extensively found in leaves and pods of senna. All across the globe it is used as excellent laxative has also been used to treat many diseases like cholera, jaundice, gout, rheumatism, tumours, typhoid, bronchitis and leprosy (Pulliah, 2002) [3]. Senna produces bright yellow flowers and is a potential crop in honeybee culture, as blooming senna provides food supply for honeybee round the year (Sharma *et al*., 1999) [4]. Senna being drought resistant medicinal legume provides not only economic profits but also a vegetational belts on the wastelands of Rajasthan where rainfall is low and erratic (Bohra, 1997) [5]. Thus, this makes the plant an ideal crop for arid regions where water provision, wasteland development, desertification control and sand dune stabilization are the major challenges (Tripathi, 1999) [6]. However, the conventional practice involves seed for multiplication of senna, but this is unreliable due to the environment-controlled dormancy of the seeds and also due to the presence of germination inhibitors (Anonymous 1992) [1]. Senna is susceptible to frost. An alternative method for mass propagation and genetic improvement is through tissue culture. Leguminosae plants are reported to be recalcitrant and quite difficult to use *in vitro* (Angeloni *et al*; 1992) [7]. There are some studies on in micropropagation of Senna (Agrawal and Sardar, 2003; Parveen *et al.*, 2012; Agrawal and Sardar 2006; Siddique *et al.*, 2010; Parveen *et al.*, 2011; Agrawal and Sardar 2007) [8-13]. But there is not a single report on micropropagation of this plant from stem derived calli. Studies related to its cultivation and genetic improvement are also very less (Jhambale *et al*., 1998; Lal *et al*., 1992) [14-15]. The current protocol is the first report on the organogenesis of Cassia *angustifolia* using stem derived callus.

Materials and Methods
Plant Material
Twigs cut from *in vivo* grown plants were dipped in Bavistin and Citric acid at their basal cut ends till they were brought to their laboratory. The twigs were defoliated and cut into approximately 1-2 cm long nodal explants. These nodal explants were dipped in 0.5% (w/v) Bavistin. These were rewashed 7 or 8 times with distilled water. After pouring out excess of water, explants were further treated with 70% alcohol for 5 mins. Subsequently, they were taken out from the solution and again rewashed with water.

Culture medium and culture conditions
The culture medium used was MS (Murashige and Skoog, 1962) [16] having sucrose (3%). This basal medium was used for the experimental purpose. Agar 0.8% were taken to prepare the medium and 0.1 N NaOH / 0.1 N HCl were used to fix the pH to 5.8, before putting the medium for autoclaving. Usually, a smaller quantity molten medium of about 25 ml was poured in each of the test tube. These culture tubes mouth was covered with cotton of non-absorbent type. These media were autoclaved for 15 minutes at1.06 kg/ cm^2 pressure to sterilize. All the cultures were raised in presence of continuous light. The photosynthetic photo flux of this light (radiated from cool fluorescent tubes) was about 45-46 W $m^{-2.}$ The temperature was fixed to approximately 25±2^0C for rearing all the cultures.

Shoot Regeneration
Healthy field grown plants of *Cassia angustifolia* were chosen. Nodal explants were excised from the twigs of these plants. These explants were reared on MS (Murashige and Skoog's 1962) medium containing 1 µM BA. Furthermore, these explants were placed vertically on culture medium to

initiate callus formation at their lower cut end. The calluses were further subculture to initiate regeneration of shoots, on MS basal medium containing various concentrations of BA, 2iPand Kinetin (0.5, 1. 5 and 10 µM)).

Rooting of shoots and plant acclimatization

Well-developed tissue cultured raised shoots of size 3-3.5 cm were taken to initiate root formation. These shoots were shifted to MS ½ strength culture medium containing sucrose (2%) having either no hormone or supplemented with different concentration of NAA (0.5 to 10µM). The plantlets were removed from the medium. These plantlets were washed thoroughly using sterilized/ autoclaved water. Later they were kept for 15 min in 0.1% Bavistin for at least 30 minutes. In the beginning these plantlets are transplanted in pots made of plastic containing Soilrite for 30 days. Polythene sheets were used to cover these plastic pots and maintained under controlled conditions like 45-46 W cm^{-2} light, relative humidity of 55-65 percentage and temperature of 25 ± 2^0C. One fourth strength MS solution were used to nourish these plantlets. This solution was without sucrose and used to irrigate the senna plantlets for one week and later with tap water, which were transferred to soil.

Observation of culture and analysis of data

Stereomicroscope were used to take the observation of cultures after seven days. All the result presented in the current study of *Cassia angustifolia* are the mean of 2 replicates.

Results and Discussion

Induction of Callus

Induction of brownish black callus at the base of nodal explant was seen on a MS culture medium consisting 5µM N^6benzyladenine, within 7-10 days of inoculation and in next twenty days quite rapidly proliferated (Figure 1A). According to Mark and Simpson (1994), [17] the reason could be the action of auxin which gets accumulated at the lower cut ends resulted in cell proliferation, particularly in the presence of the hormone cytokinins.

Shoot Formation

The brownish black, compact and morphogenic callus after excision of axillary shoots emerging from node were transferred to various levels (0, 0.5, 1. 5.10) of Kinetin, N6-bezyladenine. Within 7-10 days of transfer, some peripheral and inner cells of the brownish black callus changed to greenish colour and develop competence for organogenesis, these results in induction of multiple shoot buds and some of them further organized into shoots in next 15-20 days. Shoot organogenesis from callus, formed at the base of nodal explants has also been reported in *Tylophora indica* (Sharma and Chandel 1920), [18] *Holostemma adakodien* (Martin 2002) [19], *Peganum harmala* (Saini and Jaiwal 2000) [20]. Shoot bud formation was not seen on MS medium without plant hormones. Of the three cytokinins tried (N^6-benzyladenine, Kinetin and 2iP), the maximum shoot regeneration (5.39±0.34) was achieved on MS medium supplemented with 5µM N^6 -benzyladenine (Table1). Among the various concentration (0.5-10 µM) of Kinetin tried, MS medium containg 5µM Kn showed the best results i.e hundred percent of cultures responded and developed an average of 4.02±0.30 shoots per culture.

A significantly low response was recorded, in the percentage of morphogenic cultures as well as the number of shoots formed per culture on Murashige and Skoog's medium containing 2iP. 83.33% of the callus cultures induced organogenesis at 5µM 2iP and shoots formation was 3.10±0.38 per culture of *Cassia angustifolia*, while at its lower levels, the response was even poorer (Table 1).

Table 1: Effect of N6- bezyladenine (BA), Kinetin (Kn) and 2iP on shoot organogenesis of *Cassia angustifolia* callus after 30 days of culture.

Growth regulators (µM)			% Responding Cultures	Average No. of shoots per culture	Average shoot length (cm)
BA	Kn	2iP			
0.5	-	-	89.58^b	1.66±0.22c	1.18±0.23^c
1	-	-	100^a	2.35±0.23^b	2.06±0.40^b
5	-	-	100a	5.39±0.34^a	2.21±0.36^a
10	-	-	100^a	1.73±0.18^c	1.04±0.16^c
-	0.5		77.08^c	1.44±0.24^c	1.37±0.32^b
1			89.58^b	3.15±0.39^b	2.09±0.54^a
5			100^a	4.02±0.30^a	2.02±0.40^a
10			100^a	1.94±0.18^c	1.07±0.16^c
0.5			68.75^c	1.14±0.19^c	1.14±0.20^c
1			77.08bc	2.06±0.32b^c	1.49±0.34^b
5			83.33b	3.10±0.38^b	2.06±0.44^a
10			93.75a	1.35±0.16^a	1.01±0.13^c

*Means±SE, n=24. Values in column having the same superscript are not significantly different as determined by SAS at $p<0.05$.

Fig 1: (a) A flowering twig of *in vivo* grown *Cassia angustifolia* (b)Differentiation of multiple shoots on MS medium having 10 µM BA after three weeks of the culture.

Rooting and Plant Acclimatization

The shoots which were well-developed taken out of the culture medium and subculture to MS medium augmented with MS salts of half strength and NAA (α-naphthaleneacetic acid). MS basal alone did not help in the formation of roots. Nevertheless, on MS half strength and 10 µM NAA seventy percentage of shoots organized 3.25± 0.45 roots.

Table 2: Effect of auxin (NAA) containing MS medium on rooting of *in vitro* developed shoots of *Cassia angustifolia* after thirty days of subculture.

NAA (µM)	% Shoots producing roots	Average* number of roots developed per shoot	Average*length of root (cm)
0.5	37.50^{c}	0.60±0.17^{c}	1.22±0.29^{c}
1.0	45.00^{c}	0.93±0.22^{c}	1.81±0.46^{c}
5.0	57.50^{b}	1.75±0.31^{b}	2.55±0.65^{a}
10.0	70.00^{a}	3.25±0.45^{a}	2.13±0.59^{b}

*Mean±SE.n=24 Values in column having same superscript are not significantly different as determined by SAS at p<0.05.

The plantlets of senna after 30 days, were taken out of the culture medium. These plantlets are kept in 0.1% Bavistin for 30 minutes before shifting to Soilrite for hardening. Later, for further acclimatization transplanted to field.

Conclusions

Among the cytokinins tried BA were more responsive than kinetin which responded better than 2iP in terms of shoot multiplication per culture. On MS medium augmented with 10 µMNAA, rooting of shoots were maximum. It has been observed that when plantlets of senna are shifted to field it was effectively acclimatized. The present study will be helpful for large scale *in vitro* production of *Cassia angustifolia* to supply to the pharmaceutical industries and for genetic transformation to raise transgenic plants having higher sennosides content.

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