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Morphological Characterization and *in Vitro* Callus Induction in Ashoka [*Saraca Asoca* (Roxb.) De Wilde.] a Vulnerable Medicinal Tree

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Abstract **— A series of experiments were carried out to establish the in vitro cultures from the explants taken from the field gene bank after surface sterilization of five different genotypes collected from Tamil Nadu and Kerala. These genotypes were studied for morphological characterisation. All parts of tree *viz.*, flowers, bark and seed have medicinal property. The tree is cross pollinated species. Leaves are paripinnately compound. Inflorescence is a corymb. Corolla is gamopetalous with four petals. Fully matured pods are black in colour which contains 4 - 8 seeds. Callus induction studies were attempted using eleven explants *viz.*, meristematic shoot tip, nodal segment, internodal segment, leaf bits, axillary bud, cotyledon, embryo, seed, anther, ovary and hypocotyl from five different genotypes in three different media *viz.*, MS medium, B_5 medium and WPM medium with 2,4-D at different concentrations (0.5 to 4.0 mg /l). Genotype 4 collected from Periyakulam, explant ovary and the treatment 4 (MS+2, 4-D 2.0 mg/l) responded best for callus induction. For *in vitro* culture of Ashoka species, indirect organogenesis *i.e.*, through the callus induction was found to be the best.**

Keywords **— Ashoka, Botany, Tree, Pod, Seed, *In Vitro* Conservation.**

I. INTRODUCTION

India is considered as one of the twelve biodiversity centres, with a unique wealth of 45000 species of which 15000 to 20000 are medicinal plants. India is already a major exporter of medicinal plants, to a tune of Rs.250 crores. It is estimated that 90 per cent of medicinal plants used by Indian industry today are collected from wild thus depleting the valuable medicinal plant wealth.

A. Classification:

Kingdom	:	Plantae
Phylum	:	Tracheophyta
Division	:	Magnoliophyta
Class	:	Magnoliopsida
Order	:	Fabales
Family	:	Fabaceae
Sub family	:	Caesalpinoideae
Genus	:	Saraca
Species	:	asoca

B. Ecology and Distribution

Ashoka, *Saraca asoca (Roxb.)* de Wilde is included in the vulnerable species. International Union for Conservation of Nature and Natural resources (IUCN) listed it in the red data list as vulnerable species. Its origin is distributed in the central areas of the Deccan plateau, as well as the middle region of the Western Ghats of India and SriLanka. In India it is distributed in evergreen forests of up to an elevation of about 750 meters. It is found throughout India, especially in Himalaya, Kerala, West Bengal, Tamil Nadu and whole southern region. In Himalaya it is found at Khasi, Garo and Lussi hills and in Kerala state it is found in Patagiri, Kaikatty and Pothundi of Palakkad, Thrisur, Kollam and Kannur districts. In Tamil Nadu it is found in Kanyakumari, Theni and Coimbatore districts.

It is becoming rarer in its natural habitat, but isolated wild, Asoka trees are still to be found in the foot hills of Central and Eastern Himalayas, in scattered locations of the northern plains of India as well as on the west coast of the subcontinent. Asoka tree has many religious and literary associations in the region. It is highly valued for its beautiful appearance, colour, beautiful foliage and abundance of its fragrant flowers. It is often found in royal palace and gardens as well as close to temples throughout India.

C. Medicinal properties

The plant is source of various types of compounds which are useful for various pharmacological activities such as antimicrobial, anthelmintic, analgesic, anti inflammatory, larvicidal, antidiabetic, uterine tonic and the species has much economic importance in the sense that all plant parts such as bark, leaves, flowers, seeds etc. have medicinal properties. The bark of *Saraca asoca* has been commonly used in Indian medicine. Bark is astringent used in uterine infections. It has a stimulating effect on endometrium and ovarian tissue and useful in menorrhagia due to uterine fibroids, in leucorrhoea and internal bleeding haemorrhoids and hemorrhagic dysentery. Bark also contains an oxytoxic principle. The phyto constituents such as flavonoids, tannins and saponins in the *Saraca asoca* leaves are responsible for various therapeutic effects. Leaves are used in stomachalgia, flowers are also used as a uterine tonic, in biliousness, hemorrhagic dysentery and diabetes. In general, it is considered as best female tonic. Fruits chewed as a substitute for areca nuts. Pods make good forage and the ash of plant is good for external application in rheum arthritis.

D. In vitro conservation

The Asoka is a cross pollinated species, where seed setting is difficult and germination is also a problem. Hence it is need of the hour to conserve the plant in order

to sustain the plant diversity. As a means of *ex situ* method of conservation, tissue culture plays an important role.

In vitro clonal propagation is an efficient means of *ex situ* conservation of plant diversity and it assists in sustainable maintenance of the present day rapidly dwindling germplasm on long term basis, especially for the medicinal plants. The present research work is formulated to standardize a simple and efficient method of *in vitro* propagation through tissue culture of *Saraca asoca* (*Roxb*.) de Wilde, through different explants. Considering the above mentioned background, the present study was contemplated, to establish reproducible *in vitro* plant regeneration system in different genotypes of Asoka in appropriate nutrient medium with the following objectives: (1) Survey and collection of genotypes (2) Morphological characterization of Ashoka tree. (3) *in vitro* culture of Ashoka.

II. Materials and Methods

The present investigation was conducted at the Tissue Culture Laboratory, Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Madurai.

A. Collection of genotypes

A survey was conducted in Tamil Nadu and Kerala randomly to collect the different types in Ashoka. Fruits in five genotypes were collected from different places which on closer examination was found to represent the species. (**Table 1**).

B. Tissue culture medium

Different explants were collected from all five genotypes under study *viz.,* meristematic shoot tip (E_1), nodal segment (E_2), internodal segment (E_3), leaf bits (E_4), axillary bud (E_5), cotyledon (E_6), embryo (E_7), seed (E_8), anther (E_9), ovary (E_{10}) and hypocotyl region (E_{11}). Explant collected from each genotype/ tree was aged approximately from 5-10 years.

All the explants *viz.,* E_1 to E_{11} kept in distilled water was transferred to petri plate containing 70 per cent ethanol for 5 minutes. Then they were transferred to double distilled water and washed thoroughly. All the explants were again placed in 0.1 per cent mercuric chloride for 3 minutes and then washed thoroughly using double distilled water and kept in separate petri plates for inoculation. The nutrient media used for the study were MS medium[8], B_5 medium [3] and Woody Plant Medium (WPM) [7] Benzyl Adenine (BA), Zeatin (Zn), N^6-Benzyl Amino Purine (BAP), Kinetin (Kn) and Indole, 3-Acetic acid (IAA) for callus induction and regeneration. The observations recorded were statistically analyzed [4] using software AGRES.

III. Results and Discussion

A. Morphological characterization of Ashoka

Ashoka is a small evergreen tree with an erect trunk. The height of the tree is 7-10 metre. The tree is with many primary and secondary branches. Leaves are paripinnately compound, narrowly lanceolate, leaves grow alternately on the branches. A matured leaf is with 6-12 leaflets each with 10-25 cm in length (**Fig 1a.).** Bark is rough, grey, brown or black in colour **(Fig 1b).** Inflorescences are produced on the branching stem as well as in terminal branches. Calyx is small, petaloid (or) with two sepals, the length of the calyx ranges from 0.8-1.0 cm and the breadth ranges from 0.2-0.3 cm. Corolla is gamopetalous with four petals, the length of the corolla ranges from 1.0-1.3 cm and the breadth ranges from 0.4-0.6 cm. Androecium is with 7 stamens. Each stamen had long filament, the length of the filament ranges from 1.8-2.3 cm and the filament breadth is 0.1 cm. Anther is dark maroon in colour, the length of the anther ranges from 0.1-0.2 cm and the breadth is 0.1 cm. Gynoecium with a long style, the length of the style is from 1.0-1.3 cm and the breadth of the style is 0.1 cm. Stigma is dark maroon colour , the length of the stigma varies from 0.2-0.3 cm and the filament breadth is 0.1 cm. Flowering season is normally in February to May. **(Fig 1c).** The tree is cross pollinated, the fruit is known as pod and are formed in clusters. The matured pods are dark green and fully matured pods are black in colour, the length of the pod ranges from 12.8-18.6 cm and the breadth ranges from 3.6-5.0 cm. The fruit is flat, linear oblong tapering at both the ends. At maturity pods split open **(Fig 1e).** The matured pod contains 4 - 8 seeds. The seeds are ellipsoid, oblong compressed and brown in colour at maturity. Generally poor seed set was observed and germination of the seed also very difficult. Generally the seeds are uneven in size and shapes **(Fig 1f).** The morphological observations of leaf, flower, pod and seed are mentioned in the **Table 2.**

B. In vitro callus induction in Ashoka

The genotypes, explants, media and various levels of the phyto hormones were significantly different among themselves for callus induction percentage. The effect of 2,4-D on genotype 1, genotype 2, genotype 3, genotype 4 and genotype 5 for callus induction was studied in three different media *viz.,* MS medium, B_5 medium and WPM medium with 2,4-D at different concentrations (0.5 to 4.0 mg /l) along with a control (Basal MS medium) using meristematic shoot tip as explant. The result presented that among the three different media tried MS with different concentrations of 2, 4-D (0.5-4 mg/l) responded well for three genotypes (G_2, G_3 and G_4). Thus it revealed the superiority of MS media over B_5 and WPM media in Ashoka tissue culture. This medium has been used most frequently in many studies [2, 5] with or without modifications of MS for callus initiation, development and maintenance.

The results showed that among the three genotypes of Ashoka, a maximum callus induction of 81.91 per cent was observed with Genotype 4 at MS with 2, 4-D 2 mg/l. The genotype 4 as well as the 2, 4-D 2 mg/l treatment had proved as the single best treatment among all other combinations and it was statistically significant. The next level of callus induction was recorded by genotype 3, that was 66.47 per cent MS with 2, 4-D 2mg/l. The Genotype recorded as 65.60 per cent at MS with 2, 4-D mg/l. The responses of all the genotypes were at lower levels with 2, 4-D 0.5 mg/l. The poorest performance was recorded in the genotype 3 (23.23 per cent) of 2, 4-D 0.5mg/l. The

remaining two genotypes (genotype 1 and genotype 5) could not induce any calli even in all the explants **(Table 3).** Similarly the influence of the genotype in callus induction and plant regeneration was reported by [1]

Among the different explants tried, ovary (E_{10}) has recorded a maximum callus induction of 88 per cent at 2, 4-D 2.0 mg/l followed by meristematic shoot tip (E_1) with 81.91 per cent while the tender leaf bits (E_4) have recorded callus induction of 78.87 per cent at same concentration of media. The lowest callus induction of 29.70 per cent was recorded in the tender leaf bits in the media combination of MS with 2, 4-D 0.5 mg/l **(Table 4).** The highest value of relative growth rate of callus 16.01 was observed in the explant ovary (E_{10}) on MS with 2, 4-D (2.0 mg/l). The lowest value of relative growth rate of callus 6.14 was observed in the explant meristematic shoot tip (E_1) on MS with 2, 4-D (0.5 mg/l). The highest mean value of relative growth rate 9.65 was recorded in the genotype 4 explant ovary (E_{10}) MS + 2,4D (2.0 mg/l) (Table 5) (Fig 2) [9]. Success *in vitro* cultures largely depends on nutrition, growth regulators, variety and their interaction between the variety and medium [6]. The differential response of various genotypes proved the effects of genotypes on callus induction.

IV. Conclusion

Eight different levels of 2,4-D were tried in five genotypes using meristematic shoot tip**.** The hormonal combination of 2,4-D 2.0 mg/l recorded earliest callus induction in 32.26 days. Shoot regeneration using different explants were also not successful. Direct organogenesis was also tried in different genotypes using MS, B_5 and Woody Plant Medium with different ranges of hormones *viz.,* BA, Zn, BAP, Kn and IAA it was found difficult to regenerate the direct shoots in any of the explant and in any combination in all the three media. From the present investigation it was concluded that for *in vitro* culture of Ashoka species indirect organogenesis *i.e.* through the callus induction was found to be the best than direct organogenesis. The Genotype 4 collected from Periyakulam and the explant 10 (ovary) proved to be the best for callus induction with MS + 2,4D (2.0 mg/l).

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Author's Profile

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Dr. K. Sivasubramaniam is a veteran seed technologist whose contribution towards seed science spans 28 years which include teaching undergraduate and post graduate students, research and extension. Upon completing his B.Sc. Agriculture in 1984 from the prestigious Tamil Nadu Agricultural University, Coimbatore he immediately endeavoured to complete post graduation in his passionate subject, Seed Science and Technology, from the Department of Seed Technology in 1986**.** His vast knowledge in seed technology has helped him to groom 4 Ph.Ds and 6 M.Sc. He has also penned 7 books at national level and 32 books in vernacular language that have been well received. His dedication to his science has seen him elevated to administrative positions as Professor and Head, Agricultural Research Station, Vaigai Dam, Dean, College of Agricultural Technology, Theni and Dean, Thanthai Roever Institute of Agricultural and Rural Development, Perambalur which he currently occupies. He was also the founder Professor and Head of Department of Seed Science and Technology at Agricultural College, Madurai and also the initiator of M.Sc in Seed Technology there. He conducted several National level seminars and regional level campaigns for promoting use of quality seed. To his credit are 12 international articles, 150 scientific articles and 224 popular articles. Propensity in promoting seed science in simple language helped him to write Self Learning Material after a month long training inducted at University of Guelph, Ontario, Canada

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Table 1. Details of Ashoka Genotypes

S. No.	Genotype	Place of Collection	District	State
1.	Genotype 1	Kaliyakkavilai	Kanyakumari	Tamil Nadu
2.	Genotype 2	Poovar	Thiruvananthapuram	Kerala
3.	Genotype 3	Periyakulam	Theni	Tamil Nadu
4.	Genotype 4	Periyakulam	Theni	Tamil Nadu
5.	Genotype 5	Botanical Garden	Coimbatore	Tamil Nadu

Table 2. Morphological observations on leaf, flower, pod and seed characters in five genotypes of Ashoka

S. No.	Plant part	Genotypes									
		Genotype 1		Genotype 2		Genotype 3		Genotype 4		Genotype 5	
		Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)	Length (cm)	Breadth (cm)
1.	Leaf let	10.5	2.8	15.1	3.8	9.6	3.1	14.8	4.5	12.3	3.3
2.	Calyx	0.8	0.2	0.9	0.3	0.8	0.2	0.9	0.3	1.0	0.2
3.	Corolla	1.1	0.5	1.0	0.4	1.2	0.6	1.2	0.5	1.3	0.6
4.	Anther	0.1	< 0.1	0.2	< 0.1	0.2	< 0.1	0.2	< 0.1	0.2	< 0.1
5.	Filament	1.9	0.1	1.8	0.1	1.8	0.1	2.2	0.1	2.3	0.1
6.	Ovary	1.0	0.2	1.2	0.2	1.1	0.2	1.3	0.3	1.2	0.3
7.	Style	1.1	< 0.1	1.2	< 0.1	1.0	< 0.1	1.3	< 0.1	1.2	< 0.1
8.	Stigma	0.2	< 0.1	0.3	< 0.1	0.2	< 0.1	0.3	< 0.1	0.2	< 0.1
9.	Pod	12.8	3.6	14.9	3.8	14.7	3.8	18.6	5.0	16.3	4.2
10.	Seed	3.0	1.8	3.4	2.4	3.1	1.9	3.5	2.2	4.0	2.5
11.	Seed Weight (gm)	8.77		9.90		9.38		11.21		10.68	

Table 3. Effect of 2,4-D at different concentration on callus induction of five Ashoka genotypes using Meristematic shoot tip (E_1) as explant

Treatment / Genotype	Weight of callus (mg)									
	Control MS Basal	MS + 2,4–D (0.5 mg/l)	MS + 2,4–D (1.0 mg/l)	MS + 2,4–D (1.5 mg/l)	MS + 2,4–D (2.0 mg/l)	MS + 2,4–D (2.5 mg/l)	MS + 2,4–D (3.0 mg/l)	MS + 2,4–D (3.5 mg/l)	MS + 2,4–D (4.0 mg/l)	Mean
Genotype 2	0.0 (0.31)	25.04 (29.52)	26.51 (34.09)	38.14 (38.12)	65.60 (56.91)	48.60 (41.03)	31.44 (30.98)	25.92 (30.31)	24.33 (30.01)	31.73 (32.40)
Genotype 3	0.0 (0.31)	**23.23 (30.06)**	28.28 (33.53)	25.14 (36.31)	66.47 (56.82)	38.92 (38.59)	35.08 (35.92)	30.52 (32.12)	26.82 (28.80)	30.49 (32.50)
Genotype 4	0.0 (0.31)	30.88 (32.55)	33.47 (35.15)	51.47 (38.87)	**81.91 (67.39)**	60.34 (51.02)	40.96 (35.15)	36.98 (32.55)	33.16 (30.83)	**41.01 (36.08)**
Mean effect of hormone	0.0 (0.31)	26.38 (30.71)	29.42 (34.25)	38.25 (37.76)	71.32 (60.37)	49.28 (43.54)	35.82 (34.01)	31.14 (31.72)	28.10 (29.88)	**Grandmean 34.41 (33.66)**

(Percentage data has been transformed by Arc-sine transformation prior to analysis) CV (%) = 4.16

	SED	CD (0.05)	CD (0.01)
Genotype	0.39	0.78	1.04
Hormone	0.67	1.35	1.81
Genotype X Hormone	1.17	2.35	3.13

Table 4. Effect of 2, 4-D on callus induction using different explants of Ashoka Genotype 4 (G_4)

Treatment / Explants	Weight of callus (mg)									
	Control MS Basal	MS + 2,4D (0.5 mg/l)	MS + 2,4D (1.0 mg/l)	MS + 2,4D (1.5 mg/l)	MS + 2,4D (2.0 mg/l)	MS + 2,4D (2.5 mg/l)	MS + 2,4D (3.0 mg/l)	MS + 2,4D (3.5 mg/l)	MS + 2,4D (4.0 mg/l)	Mean
Meristematic Shoot tip (E_1)	0.0 (0.31)	30.88 (32.55)	33.47 (35.15)	51.47 (38.87)	81.91 (67.39)	60.34 (51.02)	40.96 (35.15)	36.98 (32.55)	33.16 (30.83)	41.01 (35.98)
Tender leaf bits (E_4)	0.0 (0.31)	**29.70 (31.39)**	32.41 (34.02)	48.30 (37.55)	78.87 (65.56)	59.60 (50.01)	38.59 (34.70)	33.81 (30.20)	31.88 (29.12)	39.24 (34.76)
Ovary (E_{10})	0.0 (0.31)	31.70 (33.06	35.98 (36.65)	54.51 (42.62)	**88.00 (72.22)**	68.66 (63.56)	47.01 (38.43)	38.32 (35.13)	35.21 (33.52)	**44.37 (39.50)**
Mean effect of hormone	0.0 (0.31)	30.76 (32.33)	33.95 (35.27)	51.42 (39.68)	82.92 (68.39)	62.86 (54.86)	42.18 (36.09)	36.37 (32.62)	33.41 (31.15)	**Grandmean 41.54 (36.74)**

(Percentage data has been transformed by Arc-sine transformation prior to analysis) CV (%) = 4.84

	SED	CD (0.05)	CD (0.01)
Genotype	0.39	0.77	1.03
Hormone	0.67	1.34	1.79
Genotype x Hormone	1.16	2.32	3.10

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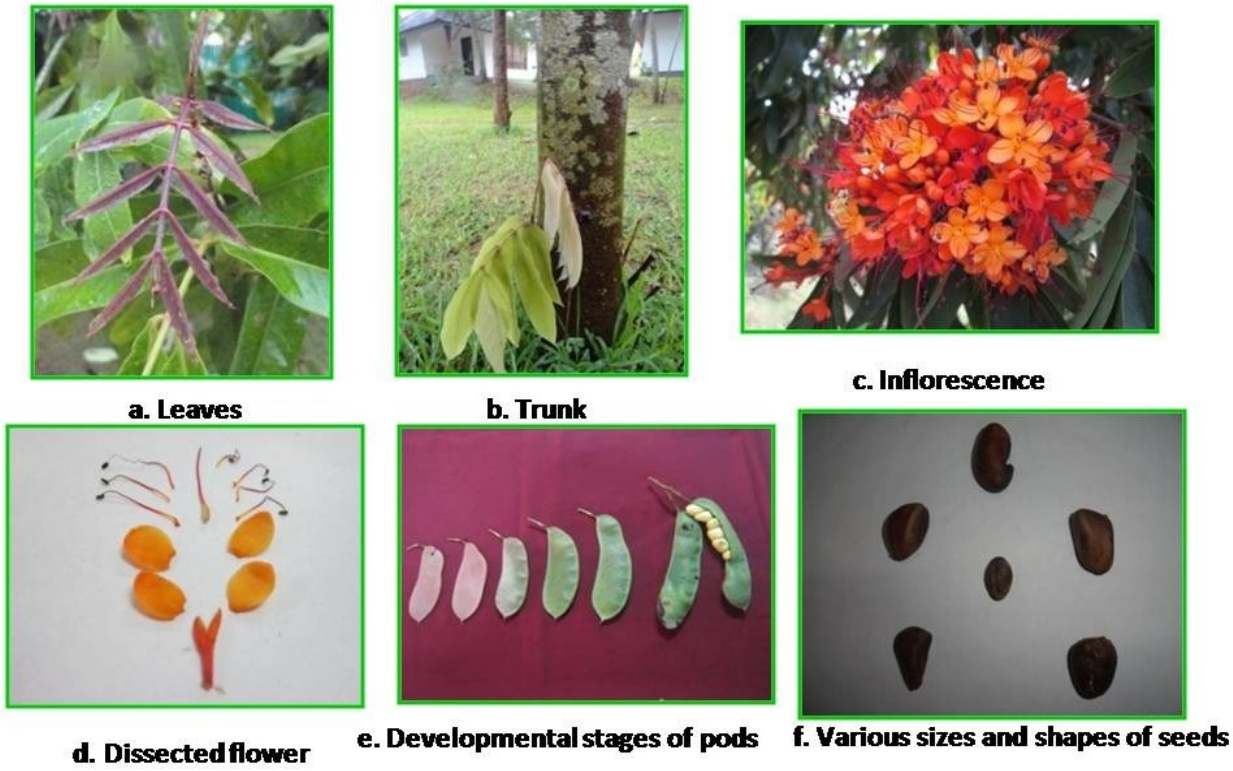
Table 5. The relative growth rate of callus on MS medium with different levels of 2, 4-D on Genotype 4 (G_4) with three explants

Treatment / Explants	Control MS Basal	MS + 2,4D (0.5 mg/l)	MS + 2,4D (1.0 mg/l)	MS + 2,4D (1.5 mg/l)	MS + 2,4D (2.0 mg/l)	MS + 2,4D (2.5 mg/l)	MS + 2,4D (3.0 mg/l)	MS + 2,4D (3.5 mg/l)	MS + 2,4D (4.0 mg/l)	Mean
Meristematic Shoot tip (E_1)	0.0	6.14	9.08	10.17	14.13	13.09	12.18	8.91	8.77	9.16
Tender leaf bits (E_4)	0.0	6.44	9.13	11.72	14.36	13.16	10.14	9.03	6.89	8.99
Ovary (E_{10})	0.0	7.31	8.36	11.56	16.01	14.05	12.87	9.52	7.13	9.65
Mean effect of relative growth rate	0.0	6.63	8.86	11.15	14.83	13.43	11.73	9.15	7.60	Grand mean 9.26

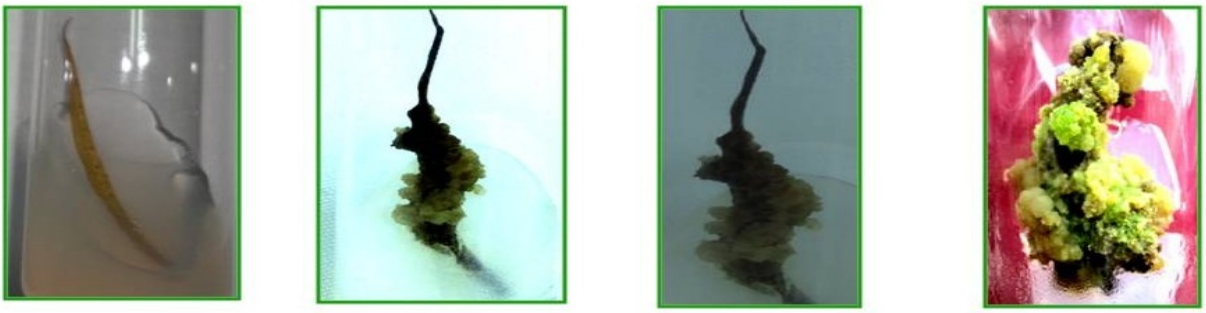
Fig 1. Morphological observations of leaf, flower, pod and seed in Ashoka



a. Leaves b. Trunk c. Inflorescence

d. Dissected flower e. Developmental stages of pods f. Various sizes and shapes of seeds

Fig 2. Callus induction from ovary



a. Bulged explant b. Callus induction c. Callus white or cream colour d. Embryogenic callus