


Successful plant regeneration system via de novo organogenesis in *Syzygium cumini* (L.) Skeels: an important medicinal tree

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Abstract *Syzygium cumini* L. (Myrtaceae) is an evergreen medicinal tree confined to tropical and subtropical regions of the world and is mainly recognized for healing of type, two diabetes. An efficient indirect regeneration system has been standardized for this tree using cotyledonary node, internode, nodal and leaf segment explants excised from 10 days old aseptic seedling. For callus induction explants were incubated on Murashige and Skoog (1962) medium supplemented with different concentrations of 2, 4-Dichlorophenoxy acetic acid (2, 4-D) or 2, 4, 5-Trichloro phenoxy acetic acid (2, 4, 5-T). All the tested concentrations facilitated callus initiation with different types and varying regeneration potentialities. The regenerated callus on different treatments showed variation in color and texture and best callogenesis (75%) was observed on (7.5 μ M) 2, 4-D after 4 weeks. Different morphogenic response was observed when callus was transferred to a secondary organogenic medium fortified with (5.0 μ M) benzyl adenine or Kinetin. The yellow white friable (YWF) callus obtained from CN explants exhibited

appearance of small leafy structures on secondary medium. Not all types of calli originating from various types of explant were organogenic. Best shoot induction (93%) were observed in (YWF) type of callus obtained from CN explants on MS medium enriched with 5.0 μ M BA + 0.25 μ M NAA + 10 mg/l AgNO₃ with maximum mean shoot number (10.19 \pm 0.05) and mean length (4.12 \pm 0.32 cm) per clump after 4 weeks of transfer. However the shoot number got increased up to 48 shoots/clump on subsequent subculturing after 12 weeks. For ex vitro rooting shoot lets (4 cm) were excised and pulse treated in full or half strength MS medium augmented with varied concentrations of IBA. Best rhizogenesis (80%) was achieved on 200 μ M IBA treated shootlets after 4 weeks of transfer to sterilized soilrite. The regenerants were successfully acclimatized and established in earthen pots with 90% survival.

Keywords De-differentiation · Recalcitrant · Phenolic exudation · Ex vitro rooting · Histology · AgNO₃

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Abbreviations

BA	Benzyl adenine
Kin	Kinetin
IBA	Indole-3-butyric acid
MS	Murashige and Skoogs medium
Min	Minute
2, 4-D	2, 4-Dichlorophenoxy acetic acid

2, 4, 5-T 2, 4, 5-Trichlorophenoxy acetic acid

Introduction

The *Myrtaceae* family comprises of valuable medicinal and economical woody aromatic species, which are enriched with essential oils and alkaloids with high antioxidant properties. *Syzygium cumini* (black plum or Indian cherry) is commercially important fruit crop cultivated in Indian subcontinent. Pertaining to its high medicinal value the plant species is in high demand in India as well as in European countries. Available pharmaceutical research across the world has shown that decoction of seeds, bark, leaves and fruits have great potential in curing the diabetes (Kumar et al. 2008; Mohamad et al. 2013). A US based patent (No. 5900240) has been approved for herbal formulation containing *S. cumini*, for diabetes treatment, (Jain and Babbar 2000). The steroids and saponins present in the leaf extract are used to treat Alzheimer disease and various neurological disorders (Alikatte et al. 2012; Ayyanar and Babu 2012). The wood is water proof and is used in making of ships, building materials, rail coaches and seats (Anonymous 2001). The seeds are difficult to store as they are prone to dehydration or desiccation and have very low dormancy period (Abbas et al. 2003; Rathore et al. 2004). Seeds have low dormancy rate and are easily susceptible to fungal infection that eventually spread endogenously throughout the plant common cultivation or orcharding practices are still not prevalent to this plant (www.agritech.tnau.ac.in). The need of the hour is to devise an efficient and economical micropropagation protocol for the conservation of this important germplasm. So application of biotechnological interventions and incorporation techniques in the forest trees species is the only promising way to be practiced for their germplasm conservation and mass scale production in short span. Establishment of reliable protocols in tree species via indirect organogenesis system has implicated success in scaling up genetic advancement and multiplication among forest and fruit trees. Major pharmaceutical companies are aiding and are deeply interested in utilizing callus culture for production of contamination free secondary metabolites on large scale. Further in many cases callogenesis has proved

best in production and synthesis of valuable alkaloids while other types of micropropagation protocols are least effective. Callus cultures could be promising possible way in understanding the regenerating process step by step via indirect organogenesis for maximum multiplication and in genetic improvement for horticultural crops (Anis and Ahmed 2016). Several authors have devised micropropagation protocols for this species through direct organogenesis (Yadav et al. 1990; Jain and Babbar 2000; Rathore et al. 2004) but regeneration via callus phase has not yet been reported. We also have earlier established a clonal propagation using mature nodal explants in *S. cumini* (Naaz et al. 2014). The present study was aimed at developing a practicable and industrial oriented regeneration protocol system via indirect organogenesis using various explants in *S. cumini*.

Materials and methods

Raising of axenic seedlings

The seeds were obtained from the fresh ripened fruits collected in the month of July and August from an elite tree growing in the University campus. These were washed thoroughly under running tap water for 20 min, treated with 5% (w/v) Bavistin (BASF-India) for 30 min, followed by 15 min immersion in freshly prepared 5% (v/v) Teepol (Qualigens, Mumbai, India). The seeds were then kept in savlon for 2 min followed by washing again and ultimately treated with 10 mg/l PVP (polyvinyl pyrrolidone) for 6 h to control phenol exudation. Surface sterilization was done with freshly prepared 0.1% (w/v) mercuric chloride (HgCl_2) solution for 3 min and finally rinsed with 3 times with sterilized double distilled water before their inoculation on different MS (full, 1/2, 1/3, 1/4) and WPM (Lloyd and Mc Cown 1981) (full or 1/2) medium for germination under culture conditions (mentioned under the sub head media and culture conditions). Cotyledonary node (CN), nodes and internodes were excised from 10 days axenic seedlings to induce callusing.

Media and culture conditions

The culture medium containing MS (1962) salts and vitamins augmented with 3% (w/v) sucrose, 0.8% (w/

v) agar (Qualigens, India) and standardized pH (5.8) was maintained before autoclaving at 121 °C for 17 min in all experiments. The gelling agent was dissolved in micro wave oven and then dispensed in borosil tubes or flasks. The basal medium was augmented with various (2.5, 5.0, 7.5 or 10.0 µM) concentrations of 2, 4-Dichlorophenoxyacetic acid (2, 4-D) or 2, 4, 5-trichlorophenoxyacetic acid (2, 4, 5-T). Incubation of explants were done in culture tubes (Borosil, India, 25 mm × 150 mm) each containing 20 ml of medium under aseptical conditions and inoculated cultures were maintained at 24 ± 2 °C with 16/8 h Photon flux density, provided by illumination of cool white fluorescent lamps (40 W, Phillips India) with an irradiance of 50 µmol m⁻² s⁻¹ in the culture room.

Callus induction and shoot bud differentiation

Efficacy of the two auxins (2, 4-D and 2, 4, 5-T) was evaluated for callus induction. Based, on the formation and consistency of callus production each auxins treatment lead to the production of organogenic calli. Since effective callogenesis was obtained in CN explants and further experiment was carried with the de-differential tissue obtained from the CN explants only. After 4 weeks of incubation, only well-developed yellow white friable (YWF) and yellow green friable (YGF) regenerating calli (3 mm pieces) from the optimal concentration of 2, 4-D (7.5 µM) were transferred to MS basal media, supplemented with two different cytokinins, 6-benzyladenine (BA) and kinetin (Kin) ranging from (0.0, 2.5, 5.0, 7.5 or 10 µM) alone or in combination with 1-naphthalene acetic acid (NAA) or indole 3 acetic acid (IAA) at concentrations (0.1, 0.25, 0.50 or 0.75 µM). The number of well-developed shoots appeared per callus was counted after 4 weeks of incubation. The adventitious shoots were sub-cultured onto the similar fresh medium after every 3 weeks. In another experiment, the effect of different concentrations (1.0, 5.0, 10, 15 or 20 mg/l) of silver ions was also evaluated in shoot bud induction and proliferation.

Ex vitro rooting and acclimatization

The basal end of the healthy shoots (4 cm) were dipped in IBA, NAA and IAA solution at concentrations ranging from (50, 100, 150, 200, 250 or 300 µM) for

30 min to induce ex vitro rooting. The pulse treated shootlets were transferred to small thermocol cups containing sterile soilrite (Keltech Energies Pvt. Ltd) covered with transparent polythene bags to ensure high humidity. Irrigation of the potted shootlets was done with half strength MS salt solution (without vitamins) for 2 weeks and data of rooting was recorded after 4 weeks. The well rooted acclimatized plantlets were transplanted to pots containing garden soil and manure (3:1) and maintained in a green house, before final transplanting to field under natural sunlight.

Histology

The histological examination was carried out in callus induced from CN explants bearing regenerated shoot buds using the protocol outlined by Fatima and Anis (2012). The callus with buds was fixed in 5:5: 90 (v/v/v) formalin: acetic acid: ethanol (FAA) for 24 h and stored in 70% (v/v) ethanol. Tissues were dehydrated with a graded ethanol–xylol series followed by paraffin embedding using the method of Johansen (1940). Longitudinal sections, approx. 10 µm thick, were cut using a Spencer 820 rotatory microtome (American Optical Corporation, Buffalo, NY, USA) and the resulting paraffin ribbons were passed through a series of alcohol grades to de-paraffinise, followed by staining with safranin and fast green (Johansen 1940). Sections were examined under a light microscope (Olympus CH20i; India Pvt., Ltd., New Delhi).

Statistical scoring

Each treatment consisted of 10 replicates and all experiments were repeated thrice. The data analyzed statistically using one way analysis of variance (ANOVA). The results were expressed as mean ± SE of 3 experiment established by Duncan's multiple range test (DMRT) at 5% ($P \leq 0.05$).

Results and discussions

Control of phenolic exudation

In-vitro seed germination and seedling growth could be an alternative method to regenerate plantlets pertaining to recalcitrant nature or decreased viable periods of seeds. *Syzygium* seeds are rich in phenolic

contents that hindered proper germination and further proliferation process and in turn affect the whole regeneration system. Phenolic compounds get oxidized to quinines by the enzymatic actions of mono phenol oxidase (MPO) and poly phenol oxidase (PPO) that destroy enzymatic activities and are inhibitory to plant cellular growth (Loomis and Battaile 1966). Therefore, it is pertinent to control the phenol exudation into the medium for successful culture establishment. Pretreatment of seeds with PVP (10 mg/l) for 6–8 h successfully control the phenolic exudation. Uses of adsorbent and antioxidants like ascorbic acid, PVP and citric acid have been successful for controlling phenolic exudation (Reynolds and Murashige 1979; Abdelwahd et al. 2006; Ahmad et al. 2013).

The right choice of planting substrates and pre-treatments given to seeds are important factors influencing germination and growth of the seedlings. MS full strength medium evoked highest and efficient seed germination with more number of nodes while WPM also evoked good germination but the length of shoots was smaller with less number of nodes than MS raised seedling along with some basal callusing in some cultures. MS full strength proved better than $\frac{1}{2}$ MS (Fig. 2). Our result corroborates earlier findings in other woody species, *Soymedafe brifuga* (Chiruvella et al. 2014).

Effect of explant type on callus induction and morphology

The initial morphogenic response was the swelling up of all the explants after 7–10 days of incubation. The explants in control medium gradually became dead within 10 days of incubation. Different explants exhibited differential response for callus induction and morphogenesis in both the auxins tested (2, 4-D and 2, 4, 5-T). All the treatments were responsive towards callus induction but the rate of growth and differentiation exhibited wide range of callogenesis, depending on the concentration of growth hormones and type of explants used (Table 1; Fig. 4). This may be attributed that certain cell layers or tissues undergo morphogenic competence with each other that later induces callus formation in only certain cells. Thus competency amongst different cellular zones results into varied callus expressions by different explants (George et al. 2008).

The best response for organogenic calli was shown by CN explants followed by internodal and nodal segments. The basal medium comprising 2, 4-D (5.0 μM) induced organogenic callus mass without any sign of embryogenesis which is in congruent to reports by Yang and Lu (2007) in *Psidium guajava* cultivars. The induced calli from 2, 4-D later manifested towards multiple shoot buds or shoots on the medium augmented with cytokinins singly (BA/Kin) or in combination treatment of cytokinins and auxins (BA/Kin + NAA) respectively. The CN explant responded with highest callusing with shoot regenerative potential. The callus type ranged from yellow white friable (YWF), yellow friable (YF), yellow green friable (YGF), green white compact (GWC), green compact (GC) in different explants (Table 1). Callus forming efficiency is concentration dependent on 2, 4-D where an increased callusing was recorded up to (7.5 μM) with 75% callus frequency in CN explant, beyond which, declination in yield was observed (Fig. 4). Induced calli at different concentrations of 2, 4, 5-T were yellow friable (YF) in CN explant and green-white compact (GWC) calli in nodal and internodal explants. These callus were less regenerative or non-organogenic (Table 1; Fig. 2b, c). The callus mass was required to be subcultured after every 2 weeks, lest they would turn brown and within subsequent weeks death of callus tissue was noticed. The induction of different types of calli from different explants could be due to ontogeny of the explants or due to some internal mechanism that takes place due to stress condition that directly or indirectly effect different anatomy of the three different explants (George et al. 2008). The nodal and internodal calli were white-greenish compact and did not show differentiation, even when transferred to different combination of auxin and cytokinins.

Histological observations and shoot differentiation from the callus

The calli derived from CN and internodal segments were transferred to MS medium containing different concentrations of BA and Kin. The yellow white friable (YWF) and yellow friable (YF) calli were more competent in getting transformed into organogenic calli than green-white compact (GWC) calli, when transferred to basal medium fortified with various concentration of BA and Kin (2.5–10.0 μM). Within

Table 1 Effect of different concentrations of Auxins on callus induction on cotyledonary node (CN), internodal and nodal segments after 4 weeks of incubation

	Auxins(μM)		%	Frequency of callusing	Texture of callus
	2, 4-D	2, 4, 5-T	Response		
Cotyledonary node					
	0.0	0.0	0.0	0.0	No response
	2.5		40	++	Yellow white friable C2
	5.0		55	+++	Yellow white friable C2
	7.5		75	+++	Yellow white friable C2
	10.0		45	++	Yellow friable C2
		2.5	26	++	Yellow white friable C3
		5.0	35	++	Yellow green friable C3
		7.5	30	+	Green green friable C1
		10.0	–	–	–
Internodal					
	2.5		30	+	Yellow green friable C3
	5.0		40	++	Yellow green friable C3
	7.5		55	+++	Yellow friable C3
	10.0		15	++	Yellow white friable C3
		2.5	25	+	Yellow friable C3
		5.0	37	+	Yellow friable C3
		7.5	30	+	Green white compact C1
		10.0	–	–	–
Nodal					
	2.5		22	+	Green white compact C1
	5.0		26	++	Green white compact C1
	7.5		30	++	Green white compact C1
	10.0		–	–	–
		2.5	15	+	Green white compact C1
		5.0	12	+	Green white compact C1
		7.5	18	+	Green white compact C1
		10.0	–	–	–

Values represents mean \pm SE. Means sharing the same letter within the columns are not significantly different ($P = 0.05$) using Duncan's multiple range test. (DMRT)

–, +, ++, +++ indicate no, slight, moderate and intense callusing

C1 non-organogenic calli, C2 good organogenic calli, C3 less organogenic calli

two weeks the YWF and YF calli got transformed into nodular calli (Fig. 1b). These calli gave rise to shoot bud like structures in most of the cultures that later differentiated into small leafy structures within 2 weeks. MS medium amended with BA (5.0 μM) induced shoot bud formation in 75% of the calli (YWF) derived from CN explant. Between the two cytokinins tested, BA was found to induce more number of shoot bud primordia as compared to Kin in CN derived callus. The efficacy of BA over Kin has been suggested by several workers in plants belonging to *Myrtaceae* family: *Syzygium jambos* (Prasanth et al. 2003); *Callistemon eviminalis* (Lin et al. 2005). However these shoot buds could not elongate even after 4 weeks of incubation (data not given). The CN

derived calli, when transferred to MS medium augmented with combination of optimal cytokinins BA (5.0 μM)/Kin (5.0 μM) and auxins (IAA and NAA) the shoot buds or leafy structures elongated into shootlets. The CN derived callus exhibited maximum shoot regeneration frequency (75%), highest shoot number (8.75 ± 0.09) and mean shoot length (3.88 ± 0.13 cm) when incubated in the basal medium comprising BA (5.0 μM) + NAA (0.25 μM) while, MS + BA (5.0 μM) + IAA (0.25 μM) exhibited a relatively low percent regeneration (55%) with mean shoot number (5.05 ± 0.04) and mean shoot length (3.25 ± 0.34 cm). Of the two auxin tested, NAA was found to be more potent than IAA for shoot morphogenesis (Table 2; Fig. 2h). It is apparent that

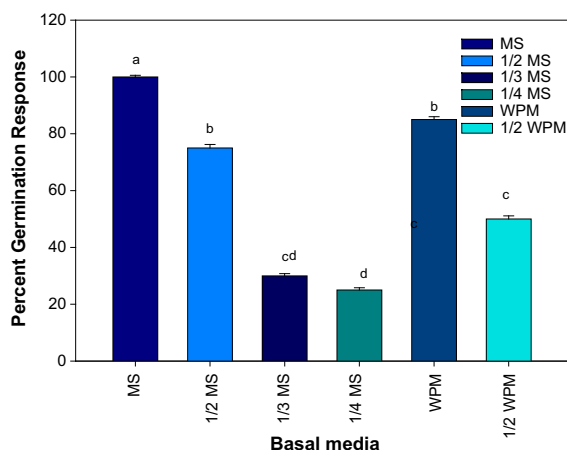


Fig. 1 Effect of different basal media on percent germination of seeds under aseptic condition. Value represents mean + SE. Mean sharing the same letter are not significantly different ($P = 0.05$) using Duncan's multiple range test. (DMRT)

cytokinins are not sufficient for shoot proliferation from callus but addition of auxins at lower concentration can trigger off shoot proliferation from the dormant shoot buds in the callus. Our report

corroborates earlier findings in *Eucalyptus* (Rodrigues and Vendrame, 2003); *Ricinus cummunis* (Kumari et al. 2008) and *Callistemon citrinus* (Raj et al. 2010). The nodal derived calli were observed to be non organogenic and could not induce any shoots in single cytokinins or in combination with auxins. This is in consonance to our finding where callus induction was observed via nodal explants but differentiation could not be achieved as in *Syzigium aromaticum* (Mathew et al. 1987). CN explants have shown superiority over other explant types for indirect organogenesis. Such differential callusing and regeneration potential has been documented well in *Populus euphratica* (Cai et al. 2015), *Citrus jambhiri* (Singh et al. 2011) and *Ricinus cummunis* (Kumari et al. 2008).

Silver nitrate (AgNO_3) has proved to be a potent inhibition of ethylene action and is widely used in plant tissue culture to promote growth and morphogenesis (Kumar et al. 2009). Addition of silver nitrate (AgNO_3) into the optimized media (MS + BA $5.0 \mu\text{M}$ + NAA $0.25 \mu\text{M}$) had positive influence up to a concentration of 10 mg/l . Maximum shoot

Table 2 Effect of optimal concentrations of different cytokinins (BA and Kin) and auxins from CN derived calli for multiple shoot induction and proliferation

Cytokinins (μM)		Auxins (μM)		% response	Mean number of shoots/clump	Mean shoot length (cm)
BA	Kin	NAA	IAA			
5.0		0.10		65	7.85 ± 0.06^b	3.58 ± 0.56^b
5.0		0.25		80	8.75 ± 0.09^a	3.88 ± 0.13^a
5.0		0.50		60	7.75 ± 0.01^b	3.66 ± 0.34^b
5.0		0.75		55	7.45 ± 0.02^{bc}	3.45 ± 0.67^{bc}
5.0			0.10	45	4.78 ± 0.06^e	3.20 ± 0.29^d
5.0			0.25	55	5.05 ± 0.04^d	3.25 ± 0.34^d
5.0			0.5	41	4.96 ± 0.09^{de}	3.21 ± 0.67^d
5.0			0.75	35	4.85 ± 0.07^e	3.19 ± 0.54^d
	5.0	0.10		28	4.75 ± 0.03^e	3.18 ± 0.21^d
	5.0	0.25		30	4.85 ± 0.04^e	3.20 ± 0.32^d
	5.0	0.5		45	5.06 ± 0.08^d	3.18 ± 0.64^d
	5.0	0.75		38	4.65 ± 0.06^e	3.14 ± 0.55^d
	5.0		0.10	35	4.05 ± 0.03^f	3.11 ± 0.29^d
	5.0		0.25	38	4.55 ± 0.03^e	3.15 ± 0.34^d
	5.0		0.5	30	4.96 ± 0.07^e	3.18 ± 0.67^d
	5.0		0.75	28	4.45 ± 0.05^{ef}	3.15 ± 0.54^d

Values represents mean \pm SE. Means sharing the same letter within the columns are not significantly different ($P = 0.05$) using Duncan's multiple range test. (DMRT)

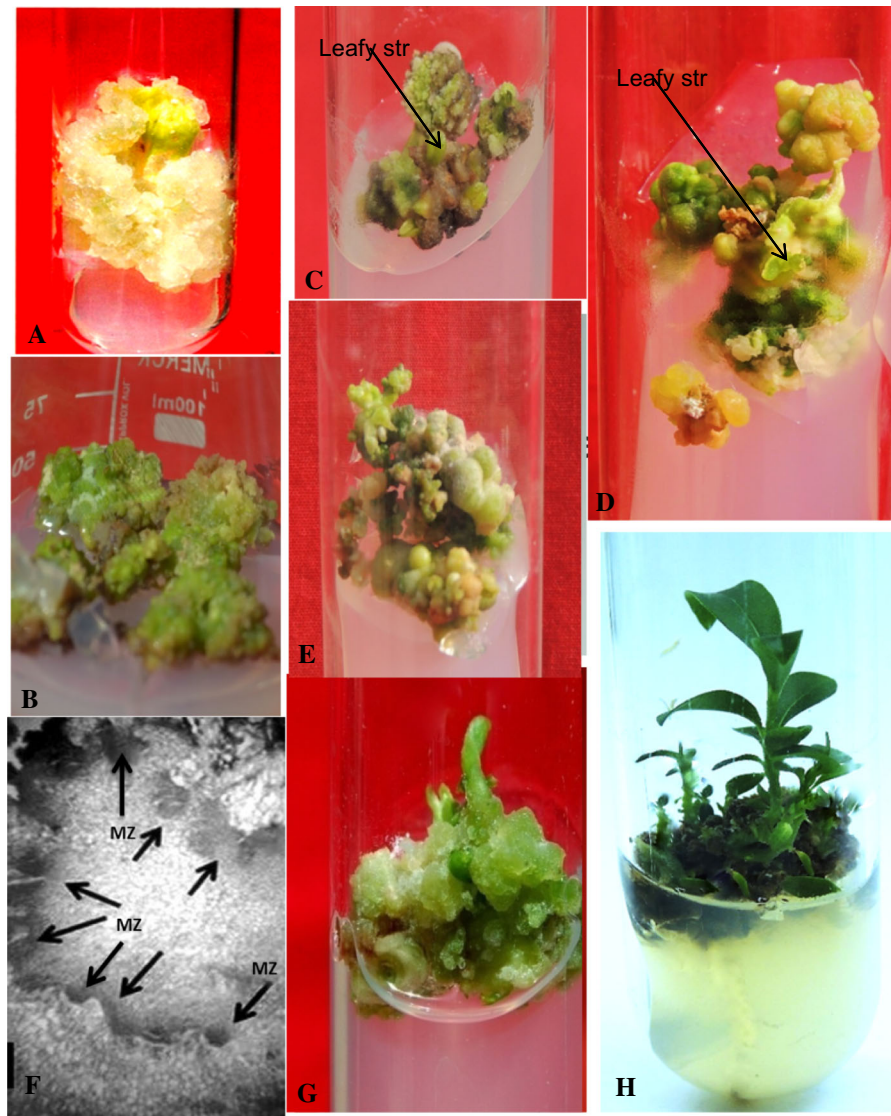


Fig. 2 **a** Callus induction from CN explant on MS + 2,4-D (7.5 μ M). **b** Induction of callus from nodal explant on MS + 2,4-D (7.5 μ M). **c** Emergence of leafy structures from internodal callus. **d** Induction of shoot buds from CN derived callus on MS + BA (5.0 μ M) + NAA (0.25 μ M) with shoot buds after 1 weeks. **e** Shoot proliferation from calli on

MS + Kin (5.0) + NAA (0.50 μ M). **f** Transverse section of callus showing meristematic zones (MZ) which gave rise to shoot primordial structures. **g** Emergence of shoots from calli on MS + BA (5.0 μ M) + NAA (0.25 μ M) after 2 weeks. **h** Adventitious shoot proliferation from CN derived callus on MS + BA (5.0 μ M) + NAA (0.25 μ M) after 4 weeks

numbers were observed to be (10.19 ± 0.56) with maximum shoot length (4.12 ± 0.32) cm, depicted in Table 3. However, beyond this concentration the callus became brown and death of shoot buds was recorded. However shoot number got increased up to 48 after 12 weeks of transfer. The positive influence of AgNO_3 up to concentration of 10 mg/l, could possibly be because of limitation of ethylene action by silver

ions due to its water soluble nature. At low concentration, silver ion mediated response seems to be involved in polyamines, ethylene and calcium mediated pathways playing a crucial role in regulating physiological process including morphogenesis (Poynton et al. 2012). However, molecular basis still need to be explored. The sharp declination in number of shoots and callus regeneration potential along with

Table 3 Effect of different concentrations of AgNO₃ on callus proliferation, and shoot regeneration with optimized medium (MS + 5.0 μM BA + 0.25 μM NAA) after 4 weeks of incubation

AgNO ₃ (mg/l)	Callus proliferation	% response	Mean number of shoots/clump	Mean shoot length(cm)	Callus texture
1.0	+	49	8.75 ± 0.09 ^c	3.88 ± 0.13 ^{bc}	Regenerative, YW, nodular
5.0	+++	71	9.89 ± 0.02 ^{ab}	3.92 ± 0.23 ^{ab}	Regenerative, YW, nodular
10.0	++++	93	10.19 ± 0.05 ^a	4.12 ± 0.32 ^a	Regenerative, YW, nodular
15.0	++	68	5.23 ± 0.04 ^d	1.05 ± 0.33 ^d	Regenerative, dried friable, browning at later stage
20.0	–	00	0.00 ± 0.00 ^e	0.00 ± 0.00 ^e	Non regenerative, dried brown callus

Values represents mean ± SE. Means sharing the same letter within the columns are not significantly different ($P = 0.05$) using Duncan's multiple range test (DMRT)

Clump = excised 3 mm callus mass

–, +, ++, +++ and ++++ , indicates no, poor, good, very good, best callusing and regeneration

drying could be due to the implication of toxic effect of silver ion from AgNO₃ on callus cells and shoot proliferation. Such toxic effect had been reported by (Bandyopadhyay et al. 1999; Sarropoulon et al. 2016). There results authenticate that the synergistic effect of AgNO₃ on shoot regeneration is species specific and concentration dependent. The internodal callus or nodal callus when transferred on MS medium augmented with BA (5.0 μM) + NAA (0.25 μM) + AgNO₃ (1, 5, 10, 15 or 20 mg/l) could not initiate shoot proliferation in any of the callus. From this study, we can explain that the callus were non regenerative and neither NAA nor AgNO₃ could trigger shoot bud induction or shoot proliferation. The callus exhibited gradual drying explaining toxicity of silver ions. It appears that addition of silver ions disrupts the ionic balance of the medium disrupting the intracellular uptake and release of free Ag⁺ ion in plants cell, sometimes causing increased oxidative stress and generation of excessive ROS, inducing toxicity and damaging cellular mechanism.

In the absence of NAA, AgNO₃ could not induce shoot elongation of the shoot buds or new shoot bud emergence (Table not shown). This observation concludes that NAA played crucial role in signal transduction. Auxin regulates various growth processes by modulating gene transcription nuclear signaling module. Auxin responses in the nucleus include transcriptional activation of auxin-regulated genes and degradation of transcriptional repressor proteins. However, concentrations above 10 mg/l show

deleterious effect on callus proliferation and shoot bud formation implicating toxic effect. The toxicity of silver ion is hypothesized to be due to disruption of membrane transport process that finally leads to the cell death.

Ex vitro rooting and acclimatization

During ex-vitro rooting, rooting rates are often higher with better root quality and chances of root damage are less. This method is often ventured to reduce the micropropagation cost and time. The best rooting hormone was observed to be IBA was found to be the effective auxin and its concentration (200 μM) produced maximum mean root number of (5.57 ± 0.18) and root length (4.60 ± 0.19 cm) per shoot, while at the concentration lesser than this, no induction of root was observed (Figs. 3a, 5a, b). Beyond the optimal level, quality and number of roots declined. At higher concentrations, IBA showed inhibitory effect on rooting. NAA treatments were less responsive than IBA. Higher concentrations (300 μM) of NAA or longer submersion duration (more than 30 min) afflicted undesirable callusing at the shoot base. However, different concentrations of IAA tested were found non-responsive for *ex-vitro* root regeneration. Our result is in consonance with earlier reports in a woody taxa *Albizia lebbeck* (Perveen and Anis 2015). In the present study the AgNO₃ regenerants exhibited better rooting up to specific concentration (15 mg/l), beyond which the rooting potential is affected



Fig. 3 a Ex-vitro rooted plantlets. b Acclimatized plants. c Hardened plants after 6 months

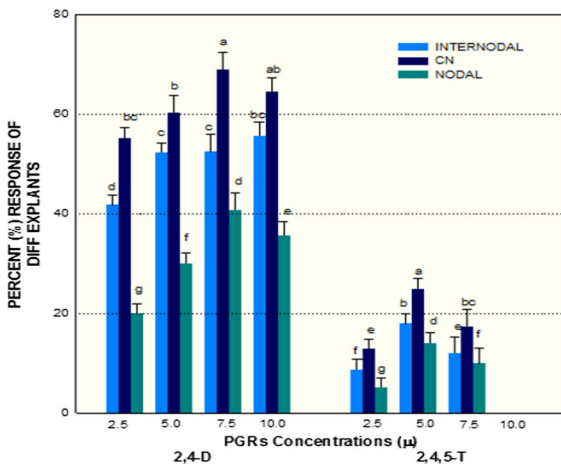


Fig. 4 Effect of different auxin concentrations on different explant obtained from axenic seedlings. The bars/lines represent mean ± SE, bars/lines denoted by the same letter variables are not significantly different (P = 0.05) using Duncan’s multiple range test

severely. Literatures have postulated that silver ions can disrupt cell water balance by inhibiting the activity of aquaporins (Niemi et al. 2002) implicating disruption in cell signaling. The toxic effect of AgNO₃ has been earlier documented in growth and

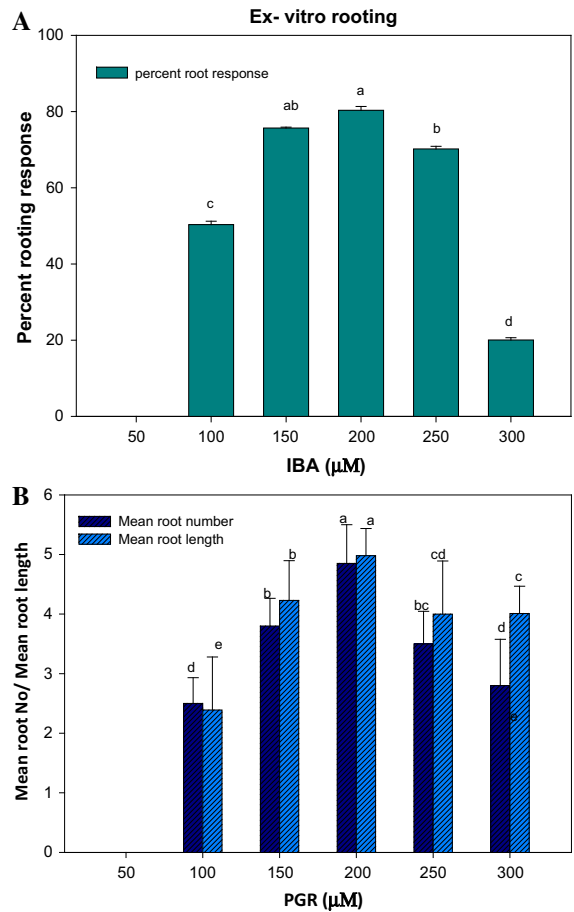


Fig. 5 a Effect of IBA for inducing ex vitro rooting in regenerants on percent rooting Fig. 5. b Effect of IBA for inducing ex vitro rooting in regenerants on mean shoot number and mean shoot length. Bars/lines represents mean ± SE. Bars/line sharing the same letter with columns are not significantly different (P = 0.05)

development of seedlings and shoots by Cvjetko et al. (2017).

Mishandling of the regenerated plantlets often restricts the commercial application of micropropagation process and wastage of expensive installation. The plants usually need some weeks of acclimatization in shade with the gradual lowering of air humidity (Pospisilova et al. 1999). The plantlets were successfully maintained in earthen pots filled with garden soil and manure (3:1), were successfully hardened inside a green house and then transferred to field conditions under open sun. A maximum of 90% survival rate was achieved as 117 plants survived out of 130 transferred plantlets (Fig. 3b).

Anatomical studies

The anatomical studies of callus tissue revealed the pattern of organogenesis via callus formation in *S. cumini*. Several meristematic zones (meristemoids) were shown in the histological sections within the callus tissue. A good number of meristemoids appeared at the periphery of calli (Fig. 2f) which is in agreement to the observation in black pepper (Sujatha et al. 2003) and in *Astercantha longifolia* (Kumar and Nandi 2015). These meristemoids frequently transformed into shoot buds leading to healthy shoots (Fig. 1f). The meristemoids situated deeply into the callus tissue remained suppressed and did not show any development. The combined effect of cytokinins and auxin resulted in enhanced shoot regeneration from organogenic calli which is in congruent with earlier studies (Ahmad et al. 2013).

Conclusion and future prospects

The difficulty of regeneration via callus still needs to be explored in trees. It is imperative that we should focus our attention on increasing our understanding on the physiological and molecular basis for the loss of regeneration potential in plant cell cultures (Fig. 4). The present study provides an alternative regeneration protocol for multiplication of *S. cumini* using cotyledonary node (CN) derived callus. The importance of the work is in the production of regenerative callus, which would be a good target to achieve genetic transformation in *S. cumini*. With the available results callus mass or suspension culture could be employed for study or production of pathogen free bioactive compound present in this species at industrial scale. The effect of heavy metal (AgNO_3) in callus regeneration provides a good insight towards tree physiology for which molecular study could be explored. The synergistic approach of tissue culture and molecular biology technique could aid in gaining the desired genetic improvement of trees.

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Author's contribution AN, SAH and RN performed the experiment, collected and analyzed data, wrote the manuscript. MA conceived and designed the experiments and edited the manuscript. AAA revised the manuscript and gave valuable suggestions.

Compliance with ethical standards

Conflict of interest The authors declare that there is no conflict of interest.

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