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Effect of plant growth regulators on high frequency callus initiation in vilvam (*Aegle marmelos* Corr.), a sacred tree species

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Abstract
Aegle marmelos (L.) Corr. commonly known as “Bael” is mostly used in Ayurveda, Unani and other traditional systems of medicine for treating various human ailments. Owing to its high therapeutic value, bael is being overexploited for the extraction of secondary metabolites. As a prerequisite for the establishment of cell suspension cultures and *in vitro* regeneration of plants, an efficient protocol for the callus induction was developed using different explants with varied hormonal concentrations of auxins (NAA, 2, 4-D) and cytokinins (BAP,KIN) either alone or in combinations. Murashige and Skoog (MS) medium supplemented with 3.5 mg L^{-1}2, 4- D alone exhibited highest callusing frequency (100%) in hypocotyl explants from *in vitro* germinated seedlings followed by 97.2% in de-embryonated cotyledon in MS media with 3.0 mg L^{-1} 2, 4-D.

Keywords: *Aegle marmelos*, callus induction, somatic embryo, multiple shoot, hormones

Introduction

India is a treasure house of various medicinal and aromatic plants. *Aegle marmelos* L. Correa, commonly known as bael achieved great importance in Indian traditional medicine due to its huge medicinal value (Kavitha *et al*., 2015) [10]. *A. marmelos* is a rich source of variety of phytochemicals including aegeline, aegelenine, marmelosine, marmelin, *o*-methyl hayordinol, alloimperratorin methyl ester, *o*-isopentenyladenine, linoleic acid, cineole, *p*-cymene, citronella, citral, cuminaldehyde, D-limonene, eugenol, tannins, phlobatannins, flovon-3-ols, leucoanthocyanins, anthocyanins and flavonoid glycoside. This wide array of phytochemicals makes *A. marmelos* an appropriate plant with neutraceutical importance (Maity *et al*., 2009) [11]. The plant is proven to possess a number of pharmacological activities such as antifungal, antibacterial, antiprotozoal, antispermatogenic, anti- inflammatory, anthelmintic, antidiabetic, laxative, febrifuge and expectorant (Narayan and Chanotia, 2009) [12].

Propagation of *Aegle marmelos* is generally through seeds which is having short viability. There will be wide changes in terms of quality and size of the fruit. *In vitro* propagation techniques can be widely used for the rapid propagation of disease free planting material within a short span of time and also, cell culture systems can be potentially used for the production of secondary metabolites of therapeutic value. Plant regeneration via *in vitro* culture techniques using different explants of *A. marmelos* has been reported i.e., nodal segments (Islam *et al.,* 2007, Puhan and Rath, 2012, Gupta *et al*., 2018) [8, 13, 6], cotyledon (Devi *et al*., 2014, Jamdhade and Pandhure, 2016) [4, 9] and leaf explants (Arumugam *et al*., 2003) [1]. Present study demonstrates an efficient protocol for the callus induction and somatic embryogenesis for the rapid multiplication of *Aegle marmelos.*

Materials and Methods

Mother plant selection

A well maintained tree of *A. marmelos* in the botanical garden of Tamil Nadu Agricultural University, Coimbatore was selected as mother plant for the collection of explants used in the *in vitro* studies.

Explant collection

The leaf, cotyledon and nodal explants were collected from the mother plant that were apparently healthy and free from any external damage of pest and pathogen attack.

They were surface sterilized and inoculated under controlled conditions. Also leaf, hypocotyl and cotyledonary explants from *in vitro* germinated seedlings were used for the callus induction studies.

Surface sterilization

Explants were treated with 2-3 drops of Tween -20 for 5 minutes, instantly rinsed with tap water and were again rinsed two times with distilled water. Further, the explants were treated with 0.1% (w/v) of $HgCl_2$ for 1 minute and then instantly rinsed three times using sterile water under aseptic condition in laminar chamber. Finally, explants were treated with 70% ethanol for 30 seconds, rinsed three times with sterile double distilled water and then dried on sterile tissue paper. Seeds were inoculated after removing the seed coat and cutting the seeds longitudinally into two halves aseptically.

Media preparation

Callus Induction Medium (CIM) was prepared from the MS media stock solutions using Murashige & Skoog (MS) nutrients supplemented with different growth regulators i.e., auxin (2, 4-D, NAA) or cytokinin (BAP, KIN) either alone or in combinations. The medium contained 3% sucrose and 0.8% agar, dissolved by boiling with constant stirring ina microwave oven. The medium was then dispensed into sterile test tubes, culture vessels were plugged with non-absorbent cotton and sterilized in an autoclave at a temperature of 121 ºC at a pressure of 15 pounds per square inch for 20 min. Heat labile growth hormones viz., kinetin, zeatin were filter sterilized using syringe membrane filters (0.2µm) and were added to the autoclaved media at a hand bearable temperature.

Plant growth regulators

Auxins

For callus initiation from various explants collected from mother plant, callus induction medium (CIM) was prepared with MS basal nutrients alone and MS medium supplemented with 2, 4- D alone at ten different concentrations (1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0 mgL^{-1}) and media is coded C1 to C11. For callus initiation from the explants derived from the *in vitro* germinated seedlings, CIM was prepared with MS basal nutrients alone and MS medium supplemented with 2,4- D alone at four different concentrations (2.5, 3.0, 3.5 and 4.0 mg L^{-1}) and the media were coded E1 to E5. For studying the effect of NAA on callus induction, MS basal media alone and MS supplemented with varied concentration of NAA(1.0, 1.5, 2.0, 2.5 and 3.0 mgL^{-1})were prepared and the media is coded N1 to N6.

Cytokinins

For studying the effect of BAP on callus induction, MS basal medium without growth regulators (B1) and MS media supplemented with BAP 1.0, 3.0 and 5.0 mgL^{-1} were prepared and coded as B2 to B4. Also, MS basal alone and MS medium supplemented with kinetin at two concentrations 1.0 and 3.0 mg L^{-1} were also tested and the media were coded K1 to K3.

Combinations of auxins and cytokinins

For studying the combined effect of auxins and cytokinins in callus induction from explants, MS basal medium alone and MS medium supplemented with combinations of BAP with 2, 4 –D and KIN with 2, 4 -D was attempted. CIM prepared with MS basal alone and MS medium supplemented with BAP and 2, 4 -D in three different combinations viz. 0.5, 1.0; 1.0, 1.0; 1.0, 2.0 mgL^{-1} were coded T1 to T4. Similarly, CIM prepared with MS basal medium supplemented with KIN and 2, 4- D in two different combinations viz. 0.5,0.5 and 0.5, 1.0 mgL^{-1} were coded R1 to R3.

All cultures were maintained at 24±2 ºC with 60% relative humidity under dark conditions. Observations were recorded on the number of explants initiating callus with the supplementation of different hormonal concentration and combinations. Sub culturing was done at 3 weeks interval to attain callus proliferation. The sub cultured plates were maintained in dark condition.

Callus induction frequency

The best treatment concentrations of the media was decided based on the callus induction frequency (expressed in percentage) observed after 30 days of inoculation.

Callus induction frequency was calculated using the following formula given below.

$$\text{Callus induction frequency} = \frac{\text{No. of explants showing initiation}}{\text{Total No. of explants inoculated}} \times 100$$

Results

Callus initiation from different explants

Seasonal variation plays an important role in determining the response of various explants towards callus initiation. Seeds collected during the month of September- November gave good response for callusing whereas those collected during the month of January did not gave any response for callusing to the same level of hormonal concentration. Among different explants viz., leaf, cotyledon from the mother plant and de-embryonated cotyledon, hypocotyl and nodal explants from *in vitro* germinated culture (Plate 1) used for determining the choice of explant for callus induction, in which hypocotyl explant gave maximum (100%) response towards callusing in the MS medium supplemented with 3.5 mg L^{-1} 2, 4- D (Plate 2). Further increase in the concentration of 2, 4-D resulted in decrease in the callus proliferation. Creamish friable calli was observed in the inoculated explants after 21 days of inoculation. Observations were recorded on the number of explants imitating callus with the supplementation of different hormonal concentration and combinations.

In case of explants obtained from the mother plant, the response towards callusing was generally very slow. De-embryonated cotyledonary explant alone responded for callus formation, whereas the leaf and nodal explants showed no response. Maximum callusing induction frequency was obtained in the cotyledonary explant and the callus proliferation was more pronounced in the MS medium supplemented with 3.0 mg L^{-1} 2, 4-D.

Effect of basal medium alone on callus induction

Cotyledonary explant did not respond towards callus induction in MS basal medium. The explants cultured in the MS basal medium for 8 weeks since inoculation, turned yellow followed by consequent browning and drying. Browning of media was observed due to exudation of phenolics from the explant.

Effect of different auxins on callus induction from cotyledonary explant

Effect of 2, 4-D alone on callus induction

Among the eleven different concentrations of 2, 4-D (MS basal, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, 5.5 and 6.0) used,

MS media supplemented with 2, 4- D 3.0 mg L^{-1} (C5) and 3.5 mg L^{-1} (C6) showed the highest callusing frequency of 97.22% whereas MS media supplemented with 6.0 mg L^{-1} 2, 4- D (C11) gave lowest callusing frequency of 38.89%. C5 is found to be the best treatment which is on par with C6. Lowest value was recorded in C11 (Table1).

The callus produced in the MS media supplemented with 3.0 mg L^{-1} 2, 4 -D started to proliferate spontaneously in the same media within one month after inoculation.

Effect of NAA alone on callus induction

Among the six different concentrations of NAA used (MS basal, 1.0, 1.5, 2.0, 2.5 and 3.0 mg L^{-1}), MS media supplemented with 2.0 mg L^{-1} (N4) gave maximum callusing frequency percentage of 36.11% whereas MS media supplemented with 3.0 mg L^{-1} (N6) gave the lowest callusing frequency percentage of 8.33%. N4 was found to be the best treatment which is on par with N3 and N2 (19.44%). N6 treatment was the poorest performing treatment (Table1).

Effect of different cytokinins on callus induction

Effect of BAP alone on callus induction

Cotyledonary explants started to develop shoots on MS medium supplemented with different concentrations of BAP hormone. Among different concentrations of BAP used(MS basal, 1.0, 3.0 and 5.0 mg L^{-1}), MS media supplemented with 3.0 mg L^{-1} (B3) gave highest callusing frequency of 36.11% whereas MS media supplemented with 1.0 mg L^{-1} (B2) gave lowest callusing frequency of 8.33%. B3 was found to be the best treatment which is comparable with B4. B2 was the poorest performing treatment (Table 2).

Effect of KIN alone on callus induction

Among the three different concentrations of KIN used (MS basal, 1.0 and 3.0 mg L^{-1}), MS media supplemented with 3.0 mg L^{-1} (K3) gave highest callusing frequency of 19.44% whereas MS media supplemented with 1.0 mg L^{-1}(K2) gave lowest callusing frequency of 8.335. K3 was found to be the best whereas K2 was found to be the poorest performing treatment (Table 2).

Effect of auxin and cytokinin combinations on callus induction

Effect of 2, 4-D in combination with BAP on callus induction

Among the different combinations of 2, 4-D and BAP used (1.0, 0.5; 1.0, 1.0 and 2.0, 1.0 mg L^{-1} respectively along with MS basal medium), MS medium fortified with 1.0 mg L^{-1} 2,4 -D and 1.0 mg L^{-1} BAP (T3) gave maximum callusing frequency of 88.88% whereas MS media supplemented with 1.0 mg L^{-1} 2,4 -D along with 0.5 mg L^{-1} (T2) gave lowest callusing frequency of 74.99% (Table 3).

Effect of 2, 4 -D in combination with KIN on callus induction

Among the different combinations of 2,4 -D and KIN used (1.0, 0.5 and 1.0, 1.0 mg L^{-1} respectively along with MS basal medium), MS medium fortified with 1.0 mg L^{-1} 2, 4-D along with 1.0 mg L^{-1} KIN (R3) gave maximum callusing frequency of 44.44% whereas MS media supplemented with 1.0 mg L^{-1} 2,4 -D and 0.5 mg L^{-1} KIN (R2) gave lowest callusing frequency of 22.22% (Table 3).

Effect of 2, 4-D on callus induction from explants of *in vitro* germinated seedlings

Effect of 2, 4 -D on callus induction from hypocotyl explant

Among the different concentrations of 2, 4 -D (MS basal, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}) used for callus induction from hypocotyl explants, MS medium supplemented with 2, 4-D 3.5 mg L^{-1} (E4) gave highest callusing frequency percentage of 100% whereas MS medium fortified with 2, 4 -D 4.0 mg L^{-1} (E5) was the poorest performing treatment (Table 4).

Effect of 2, 4 –D on callus induction from cotyledonary explant

Among the five different concentrations of 2, 4 -D (MS basal, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}) used for callus induction from cotyledonary explants, MS medium supplemented with 2, 4-D 3.0 mg L^{-1} (E3) gave highest callusing frequency percentage of 74.99% whereas MS medium supplemented with 4.0 mg L^{-1} (E5) was found to be the poorest performing treatment with a callusing frequency percentage of 33.33% (Table 4).

Effect of 2, 4 -D on callus induction from nodal explant

Among the five different concentrations of 2, 4 -D (MS basal, 2.5, 3.0, 3.5 and 4.0 mg L^{-1}) used for callus induction from nodal explants, MS medium supplemented with 2, 4-D 2.5 mg L^{-1} (E2) and 3.0 mg L^{-1} (E3) gave callusing frequency percentage of 2.77% whereas MS medium supplemented with 3.5 and 4.0 mg L^{-1} (E4 and E5) did not showed any response (Table 4).

Determination of growth of cells in callus cultures

Dry weight percentage was calculated for the treatments with maximum callus induction frequency percentage at an interval of 7 days. An increase in the dry weight was observed in both the treatments upon each subculture (21, 42 and 63 days). The cotyledon derived callus cultures in the MS medium supplemented with 3.0 mg L^{-1} 2,4-D gave a dry weight percentage of 9.62% whereas hypocotyl derived callus cultured in the MS medium supplemented with 3.5 mg L^{-1} 2, 4-D gave dry weight percentage of 8.06% (Table 5).

Table 1: Effect of auxins alone on callus induction from cotyledonary explant of *Aegle marmelos* (L.) Corr**.**

MS basal medium supplemented with concentrations of auxin (mg/l)		Callusing frequency % Mean ± SE
2, 4 -D	**NAA**	
MS basal	**MS basal**	0
1.5	-	55.55 ± 2.776
2.0	-	72.22 ± 7.349
2.5	-	80.55 ± 7.349
3.0	-	97.22 ± 2.780
3.5	-	97.22 ± 2.780
4.0	-	80.55 ± 7.349
4.5	-	72.22 ± 7.349
5.0	-	63.89 ± 7.349
5.5	-	55.55 ± 7.349
6.0	-	38.89 ± 7.349
-	1.0	19.44 ± 7.349
-	1.5	19.44 ± 2.780
-	2.0	36.11 ± 7.349
-	2.5	22.21 ± 5.556
-	3.0	8.33 ± 4.809

Table 2: Effect of cytokinin alone on callus induction from cotyledonary explant of *Aegle marmelos* (L.) Corr.

MS basal medium supplemented with concentrations of cytokinin (mg/l)		Callusing frequency %
BAP	**KIN**	**Mean ± SE**
MS basal	**MS basal**	**0**
1.0	-	8.33 ± 4.809
3.0	-	36.11 ±7.349
5.0	-	13.88 ±7.349
-	1.0	8.33 ± 4.809
-	3.0	19.44 ± 2.780

Table 3: Effect of auxin and cytokinin on callus induction from cotyledonary explant of *Aegle marmelos* (L.) Corr.

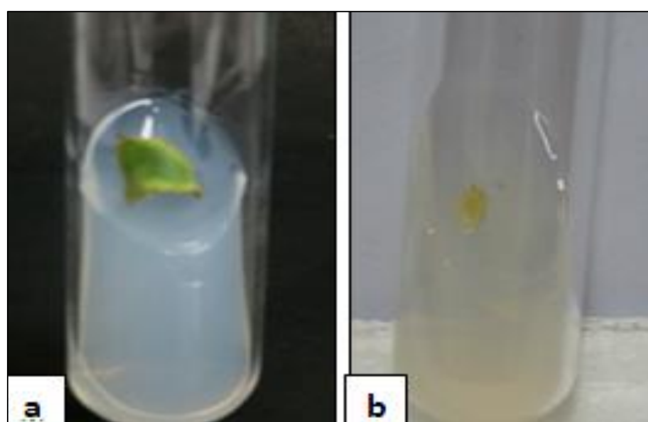
MS basal medium supplemented with concentrations of PGR's (mg/l)			Callusing frequency %
2, 4-D	**BAP**	**KIN**	**Mean ± SE**
MS basal	MS basal	MS basal	0
1.0	0.5	-	74.99 ± 8.333
1.0	1.0	-	88.88 ± 7.349
2.0	1.0	-	83.33 ± 4.809
1.0	-	0.5	22.22 ± 7.349
1.0	-	1.0	44.44 ± 7.349

Table 4: Effect of 2, 4 -D alone on callus induction from the explants from *in vitro* germinated seedlings

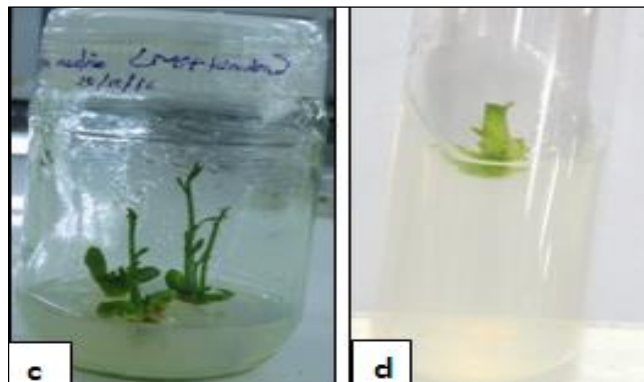
MS medium fortified with 2, 4-D alone (mg/l)	Explant used for callus induction		
	Hypocotyl Callusing frequency % Mean ± SE	**Cotyledon Callusing frequency % Mean ± SE**	**Node Callusing frequency % Mean ± SE**
MS basal	0	0	0
2.5	63.88 ± 12.109	61.11 ± 7.349	2.77 ± 2.776
3.0	72.22 ± 7.349	74.99 ± 4.812	2.77 ± 2.776
3.5	100 ± 0.00	36.10 ± 2.776	0
4.0	47.22 ± 7.349	33.33 ± 14.434	0

Table 5: Determination of growth of cells in callus cultures

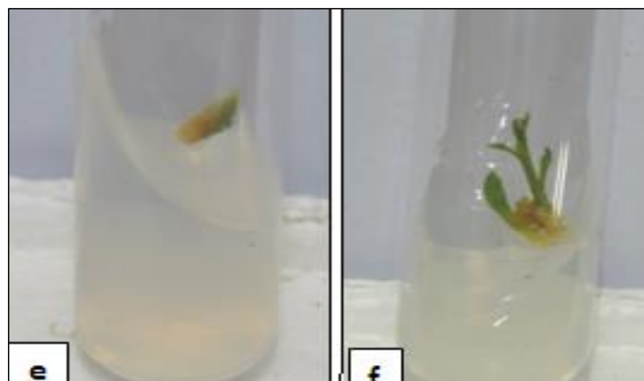
Passage (Days)	Dry weight percentage (%) of callus culture							
	Cotyledon derived callus @ MS + 3.0mg/l 2, 4-D				Hypocotyl derived callus @ MS + 3.5 mg/l 2, 4-D			
	R1	**R2**	**R3**	**Mean ± SE***	**R1**	**R2**	**R3**	**Mean ± SE***
I (21days)	6.12	5.89	6.03	6.01±0.066	5.26	4.95	5.13	5.11±0.089
II (42 days)	7.69	7.93	7.93	7.90±0.116	7.27	6.89	6.97	7.04±0.115
III (63 days)	9.87	9.46	9.46	9.62±0.125	8.33	7.95	7.91	8.06±0.133



a) Leaf b) Cotyledon

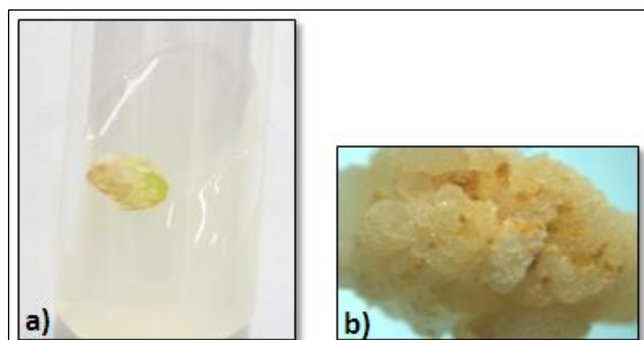


c) *In vitro* germinated seedlings d) Hypocotyl

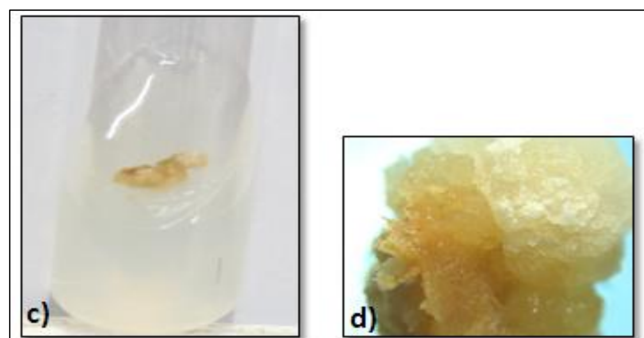


e) Cotyledon from *in vitro* germinated seedling f) Node

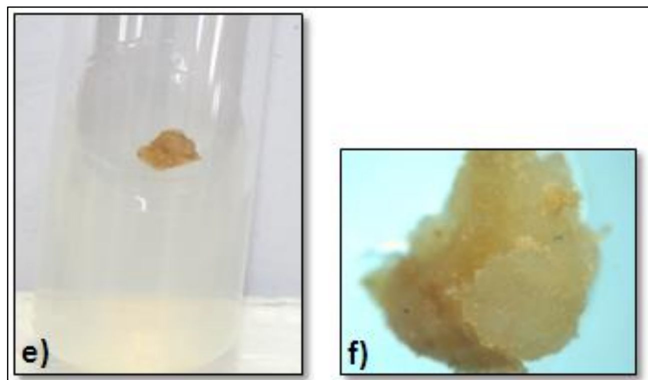
Plate 1: Different explants used for callus induction



a) 3 weeks after inoculation in callus induction medium (CIM- MS + 3.0 mg L^{-1} 2, 4-D) b) 6 weeks after inoculation in CIM (MS + 3.0 mg L^{-1} 2, 4-D)



c) 3 weeks after inoculation in CIM (MS + 3.5 mg L^{-1} 2, 4-D) d) 6 weeks after inoculation in CIM (MS + 3.5 mg L^{-1} 2, 4-D)



e) 3 weeks after inoculation in CIM (MS + 2.0 mg L^{-1} NAA)

f) 6 weeks after inoculation in CIM (MS + 2.0 mg L^{-1} NAA)

Plate 2: Effect of auxins on callus induction from cotyledon explants of *A. marmelos*

Discussion

An efficient *in vitro* plant regeneration from the callus culture is essential for the mass propagation and has advantages over other methods of plant regeneration and is useful in several manipulation studies including genetic transformation where direct regeneration may not be effective (Faisal *et al.,* 2006) [5]. In the present study, callus was induced from the cotyledonary explants in MS media supplemented with different auxins *i.e.* 2, 4-D and NAA, cytokinins such as BAP and KIN either alone and in combinations.

Exogenous application of auxin and cytokinin induces callus in various plant species. An intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin to cytokinin or vice versa induces root or shoot elongation respectively (Skoog and Miller, 1957) [14].

Among the auxins used for the callus induction from the cotyledonary explant, 2, 4-D was found to be the best and the findings has been evidenced from the results of Arya *et al.* (1981) [2] in *A. marmelos*. 2, 4-D at 3.0 and 3.5 mg L^{-1} gave highest callusing frequency of 97.2% followed by 2.5 mg L^{-1} and 4.0 mg L^{-1} with a callusing frequency of 80.55%. The callusing frequency reported in the present study is relatively higher than similar results reported by Das *et al*. (2009) [3] where creamish colour friable calli was obtained at a concentration of 4.0 mg L^{-1} 2, 4-D with a callusing frequency of 80%. The increased frequency may be attributed to the nature of the explants and seasonal variations in explant collection from the mother plants. Islam *et al.* (1995) reported similar findings that 2, 4-D, lAA and NAA induced callus and 2, 4-D was found to be more efficient for induction of callus from mature and immature embryo axis of *A. marmelos.*

Among the cyokinins used, BAP was found to be more responsive than KIN where highest callusing frequency of 36.11% was obtained using 3.0 mg L^{-1} BAP. Among the auxins and cytokinin combinations tested *i.e.* 2, 4-D with BAP and 2, 4-D with KIN, 2, 4-D in combination with BAP at 1.0 mg L^{-1} each was found to be more responsive to callus formation with a callusing frequency of 88.88% followed by 2.0 mg L^{-1} 2, 4 -D and 1.0 mg L^{-1} KIN with a callusing frequency of 83.33%. The present findings corresponds with the reports of Yadav and Singh (2011) [17], where callusing frequency of 80% was observed from the nodal explants when cultured in MS medium supplemented with BAP 2.0 mg L^{-1} and 2, 4-D 1.0 mg L^{-1} concentrations.

The combined effect of BAP 10 µM + NAA 0.5 µM elicited callus formation in 40.3% explants of both mature and immature embryo axis. In contrast Varghese *et al.* (1993) [15] reported that NAA (5.0 mg L^{-1}) + KIN (1.0 mg L^{-1}) combination produced 95.2% callus response in *A. marmelos.* Effect of KIN supplementation in increasing the callusing frequency has been reported by Hossain *et al*. (1994) [7]. The present study has distinct advantages over previous reports that requirement of only one auxin 2, 4-D individually at a concentration of 3.0 mg L^{-1} was enough for maximum callus induction than the all auxins or cytokinin either alone or in combinations.

In our present study, among the different explants used for callus induction such as hypocotyl, cotyledon and nodal explants from the *in vitro* germinated seedlings (4 weeks old) hypocotyl was found to be most responsive for callus induction and the results corroborates with the reports of Arya *et al.* (1981) [2]. The differences in callusing ability of various explants are attributed to their physiological status (Vasil and Thorpe, 1994) [16].

Conclusion

The result obtained from our study indicates that the choice of the explant and hormonal concentration plays an important role in the callus induction from Bael, a recalcitrant tree species. The morphology of the embryogenic calli obtained will determine the somatic embryo formation. Callus thus obtained can be further used for the regeneration of plantlets via indirect organogenesis and can also be used for the establishment of cell suspension cultures in this plant having immense medicinal value.

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