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In vitro regeneration and extraction of secondary metabolites in *Aegle marmelos* (L.) Correa

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ABSTRACT

Aegle marmelos is an important ayurvedic medicinal tree belonging to the family of Rutaceae. A.marmelos is used in the treatment of various diseases like cholera, dysentery, gastroenteritis and diabetes. The present study was carried out to develop an efficient in vitro regeneration system via callus phase by using various plant growth regulators and to identify the presence of potent medicinal compounds in the callus extracts of the plant. The effects of various plant growth regulators (Auxins and Cytokinins) in plant growth and the best organic solvents for the extraction of medicinal compounds were studied. The nodal explants of A. marmelos were cultured on MS medium containing various concentrations of auxins (IAA, IBA and 2,4-D) in combination with 0.5 mg/l of BA for callus induction. Among them 2,4-D (1.5 mg/l) responded well by giving the maximum percentage of callus induction (86%). The 5-6 weeks old Green compact nodular (GCN) callus was transferred to shoot induction medium (SIM) containing MS + BA (1.5 mg/l) + 2,4-D (0.2 mg/l) and produced maximum of 9-10 shoots / explant. The shoots were elongated [maximum shoot length-8.2cm] using MS + GA_3 (0.5mg/l) + BA (1.5 mg/l), where as IBA (1.5mg/l) produced maximum no of roots (9-10 roots/explant). The rooted plants were hardened in mist chamber at a survival rate of 75%. For the extraction of secondary metabolites (medicinal compounds), well developed (5-6 weeks old) Green compact nodular callus tissue (approximately 200 mg dry weight) was dried at room temperature and extracted with organic solvents such as Acetone, Methanol, Benzene, Petroleum ether and Chloroform. The presence of the medicinal compounds such as alkaloids, Flavonoids, Phenolic Compounds, Steroids, Saponins and Xanthoproteins were detected.

Keywords: *Aegle marmelos*, Regeneration, 2, 4-D, BA, IBA, organic solvents

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INTRODUCTION

Aegle marmelos is an important endangered, ayurvedic medicinal tree belonging to the family Rutaceae (Singh, 1985). It is a medium sized, armed, aromatic, deciduous tree. It is a native (Fig-1) of the Indo- Burma Subcontinent (Hooker, 1975) and is now cultivated in India, Pakistan, Bangladesh, Srilanka, Myanmar and Thailand (Zaman, 1988). Various parts including fruits possess medicinal properties and have been extensively used in ayurvedic and folk medicine (Biswas and Gosh, 1973; Sharma, 1980). The fruit, round, pyriform, oval or oblong is dotted with aromatic, minute oil glands. A decoction of the unripe fruit, with fennel and ginger, is prescribed in cases of hemorrhoids. The psoralen in the pulp is employed in the treatment of leukoderma. Bitter, light- yellow oil extracted from the seeds is given as a purgative (Khan and Haq, 1975). The plant has the capacity to adapt successfully in a wide range of habitat ranging from arid, semi- arid to mesophytic conditions (Arya, 1986).

Tissue culture technology has proved successful in the multiplication and conservation of this medicinal and aromatic plant.

Plant regeneration from nucellar tissues of *Aegle marmelos* through organogenesis was achieved by Hossain *et al*., 1993. Rapid clonal multiplication was also done through *in vitro* axillary shoot proliferation of *Aegle marmelos* (Ajithkumar and Seeni, 2004). In micropropagation, complete plantlets were produced from the cotyledons of *Aegle marmelos* (Islam *et al*., 2006)

Therefore, in the present study an attempt has been made to standardize a protocol for *in vitro* regeneration of *Aegle marmelos* from the nodal explants and to screen for the presence of bioactive fractions in the plant.

MATERIALS AND METHODS

Healthy and young shoot cuttings of *Aegle marmelos* each bearing one to two nodes were collected from 24-year-old tree. After removing the leaves, the nodal segments were washed thoroughly under running tap water for 20 minutes followed by treatment with Teepol solution for 5 minutes and another wash with distilled water. Under aseptic conditions, the nodes were then surface sterilized with 0.1% mercuric chloride for 5 minutes and washed 3-4 times with sterile distilled water. After surface sterilization, the explants were cultured on MS medium (Murashige and Skoog, 1962) containing 3% (w/v) sucrose (Himedia, India), various concentrations of cytokinins and auxins (Himedia, India). The pH of the medium was adjusted to 5.8 before gelling with 0.8% (w/v) agar (Himedia, India) and autoclaved at 121^0 C (15 lbs) for 20 mins.

The 25X150 mm culture tubes (Borosil, India) were used for each combination of growth hormone. All cultures were maintained in a growth room under 16 h photoperiod at 25 ± 2^0 C. All experiments were repeated twice.

Callus induction
The nodal explants were cultured on MS basal medium supplemented with various concentrations of IAA, IBA and 2,4-D (0.5-2.5 mg/l) and BA (0.5 mg/l) for callus induction. The data was taken 45 days after culture initiation.

Optimization of shoot regeneration
The morphogenic calli (Green compact nodular callus approximately 200 mg) obtained from the nodal explants were separated from all the treatments and transferred to MS basal medium containing different concentrations of BA, Kn and TDZ (0.5-2.5 mg/l) and 2,4-D (0.2 mg/l) for shoot regeneration. The frequency of regeneration and the number of shoots in each treatment were calculated 35 days after subculture.

Multiplication and elongation of shoots
Clusters of regenerated shoots attached to the loose callus from the nodal explants were separated and transferred to MS basal medium containing different concentrations of GA_3 (0.1-1.0 mg/l) and BA (1.5 mg/l) for further elongation and multiplication of shoots.

Rooting and hardening
The elongated shoots were transferred to half-strength MS medium fortified with different concentrations of IAA, IBA and NAA (0.5-2.5 mg/l) for root induction. Plantlets with well developed roots were removed from the culture tubes and after washing the roots in running tap water, they were grown in the mixture of red soil, sand and saw dust in 1:1:1 ratio in the plastic cups for 15 days and subsequently transferred to pots.

Statistical analysis
All experiments were repeated 2 times with 5 replicates. Data were recorded at 15 days interval. Data on multiple shoot production and rooting were statistically analyzed by Analysis of Variants (ANOVA) using the SAS system for Microsoft windows, Release 6.10. Significant differences between means were determined at p=0.05 level of significance using Duncan's Multiple Range Test (DMRT).

Extraction of bioactive compounds
The well-developed 5-6 weeks old callus (approximately 200 mg dry weight) was dried at room temperature (39˚C) and made into powder. For further analysis various organic solvents (Acetone, Methanol, Benzene, Chloroform and Petroleum Ether) were used. Extracts of dried callus powder through the Soxhlet apparatus were used to test the

presence or absence of secondary metabolites such as steroids, triterpenoids, alkaloids, tannins, phenolic compounds, saponins, xanthoproteins, flavonoids, essential oils, volatile oils and carbohydrates by various standard phytochemical methods.

RESULTS AND DISCUSSION

A series of attempts were made for the rapid organogenesis of *Aegle marmelos* from the nodal explants (3-4 cm) using different auxins (IAA, IBA, 2, 4-D) and Cytokinins (BA, Kn and TDZ) either individually or combined.

Effect of auxins and Cytokinins combination

Presence of various concentrations of auxins (IAA, IBA, 2, 4-D – 0.5-2.5 mg/l) + BA (0.5 mg/l) in the MS medium promoted callusing from the basal part of the explant. Initially the callus was friable and yellowish in color, but later it became green, compact and nodular [MS + 2,4-D (1.5 mg/l) + BA (0.5 mg/l)]. Of all the combinations tried, MS + 2,4-D (1.5 mg/l) + BA (0.5 mg/l) was the best (86%) in terms of producing viable organogenic GCN callus from the basal part of the explant (Table-1, Fig-2A).

When this organogenic callus was transferred to MS medium containing BA (0.5 mg/l) + 2,4-D (0.2 mg/l), only four shoots proliferated. However on increasing the concentration of BA (1.5 mg/l), maximum number of shoots (10 shoots/ explant) was regenerated (Table-2, Fig-2B). Further increasing of the concentrations of BA upto 2.5 mg/l did not favour the regeneration of shoots but seems to depress. The other two cytokinins Kn and TDZ of the concentration 1.5 mg/l when supplemented to the medium along with 0.2 mg/l of 2,4-D, nearly 6 and 7 shoots regenerated respectively. The present results are in agreement with Rao *et al* (1973) that high concentration of auxins and Cytokinins in low concentration was more effective for callus formation in *Petunia* sps. Similar results were observed in groundnut by Venkatachalam and Jayabalan (1997).

A survey of literature shows that high concentrations of auxins as well as Cytokinins were used for callus induction, but a maximum of 75% callusing was observed only after a month. The present investigation has some distinct advantage over previous reports that 1.5 mg/l 0f 2,4-D and 0.5 mg/l of BA itself is sufficient for maximum frequency of callusing (86.0%).

In the above cultures, callus formation and shoot regeneration could be greatly increased by regular subculturing.

Effect of GA_3 in shoot elongation

MS + various concentrations of GA_3 (0.1-1.0 mg/l) + BA (1.5 mg/l) was tried for the elongation of the regenerated shoots. On minimum concentration of GA_3 (0.10 mg/l), the shoot elongated to 4 cm. The regenerated shoots, when transferred to MS medium containing GA_3 (0.5 mg/l) in combination with 1.5 mg/l BA, elongated to 8.2 cm. The maximum response (88.2%) with maximum number of shoots (5.5 shoots/explant) was observed in the above media composition (Table-3, Fig-2C). On further increasing the concentration of GA_3 upto 1.0 mg/l, the elongation of the shoots was found to be limited.

Rooting of the regenerated shoots

The regenerated and elongated shoots thus obtained from the above cultures were transferred to half-strength MS supplemented with various concentrations of auxins (IBA, IAA and NAA). Half-strength MS + IBA (1.5 mg/l) was found suitable for the formation of healthy roots from the basal parts of the shoots (Table-4, Fig-2D). 1.5 mg/l of IBA produced the maximum number of roots (9.8) with root length of 6.7 cm.

Hardening

Rooted plantlets were thoroughly washed in running tap water to remove agar and transferred to pots containing red soil:sand:saw dust in the ratio of 1:1:1 (Fig-2E). The survival rate of the plantlets was nearly 75%. The hardened plants were then subsequently transferred to the field.

Extraction of bioactive compounds

Secondary metabolites were extracted from the dried powder of the callus by using various organic solvents like benzene, petroleum ether, chloroform, acetone, methanol and the presence of the bioactive compounds such as

alkaloids, flavonoids, phenolic compounds, steroids, saponins and xanthoproteins were detected (Table-5) by the standard methods such as Libermann-Buchard reaction.

RESULTS

Table-1 Effect of different concentrations of IAA, IBA and 2, 4-D on callus induction from the nodal explants of *Aegle marmelos* in MS medium fortified with BA (0.5 mg/l)

Concentration of Plant Growth Regulators (mg/l)	Percentage of callus induction	Nature of Callus
2,4-D		
0.5	26.8	YGF
1.0	54.4	YGF
1.5	86.0	GCN
2.0	64.5	YGC
2.5	44.6	YBC
IBA		
0.5	15.0	YGF
1.0	35.6	YGF
1.5	62.2	GCN
2.0	40.2	YBC
2.5	31.4	BGC
IAA		
0.5	17.2	YGF
1.0	37.6	YGF
1.5	64.6	GCN
2.0	43.2	YBC
2.5	32.2	BGC

Y: Yellow; G: Green; B: Brown; C: Compact; N: Nodular, F: Friable

Table-2 Effect of different concentrations of BA, Kn and TDZ on regeneration of shoots from the callus in MS medium fortified with 2,4-D (0.2mg/l)

Concentration of Plant Growth Regulators (mg/l)	Percentage of response	Mean number of shoots
BA		
0.5	60.0	4.66±0.11 h
1.0	73.2	6.09±0.71 ef
1.5	86.6	9.88±0.12 a
2.0	71.6	6.78±0.15 c
2.5	53.2	5.35±0.10 g
Kn		
0.5	51.2	4.15±0.14 i
1.0	60.0	5.15±0.15 g
1.5	71.8	6.48±0.12 d
2.0	63.2	5.90±0.14 f
2.5	54.2	4.47±0.15 h
TDZ		
0.5	53.5	5.23±0.21 g
1.0	62.6	6.15±0.20 ef
1.5	74.2	7.42±0.15 b
2.0	67.2	6.33±0.12 de
2.5	51.6	5.85±0.10 f

Values represent the treatment means of 5 replicates

Values with the same letter within columns are not significantly different according to Duncan's Multiple range test (DMRT) at 5% level

Table-3 Effect of various concentrations of GA_3 on elongation of regenerated shoots in MS medium fortified with BA (1.5 mg/l)

Concentration of Plant Growth Hormone (mg/l)	Percentage of Response	Mean shoot length (cm)	Mean number of shoots
GA_3			
0.10	53.2	4.13±0.12 d	2.03±0.14 d
0.30	64.0	6.35±0.24 b	3.20±0.17 b
0.50	88.2	8.23±0.21 a	5.45±0.19 a
0.75	81.4	6.37±0.12 b	3.17±0.10 b
1.0	78.2	5.22±0.24 c	2.78±0.13 c

Values represent the treatment means of 5 replicates

Values with the same letter within columns are not significantly different according to Duncan's Multiple range test (DMRT) at 5% level

Table-4 Effect of various concentrations of IBA, IAA and NAA in MS medium for induction of roots from the regenerated shoots

Concentration of Plant Growth Regulators (mg/l)	Percentage of response	Mean root length (cm)	Mean number of roots
IBA			
0.5	77.6	5.18±0.25 f	7.16±0.18 d
1.0	87.6	6.10±0.31 c	8.37±0.54 c
1.5	92.0	7.43±0.21 a	9.87±0.15 a
2.0	80.6	5.33±0.15 ef	8.65±0.10 b
2.5	72.2	4.45±0.15 g	6.32±0.12 f
IAA			
0.5	42.2	4.25±0.23 g	5.20±0.17 h
1.0	53.2	5.18±0.16 f	6.40±0.14 ef
1.5	60.0	5.98±0.58 c	7.32±0.20 d
2.0	52.2	5.25±0.26 f	6.65±0.25 e
2.5	40.2	4.25±0.21 g	5.40±0.17 h
NAA			
0.5	44.2	4.27±0.25 g	5.67±0.12 g
1.0	54.2	5.85±0.14 cd	6.50±0.14 ef
1.5	64.0	6.78±0.23 b	8.42±0.15 bc
2.0	53.2	5.62±0.22 de	6.57±0.12 ef
2.5	42.8	4.42±0.15 g	5.42±0.16 h

Values represent the treatment means of 5 replicates
Values with the same letter within columns are not significantly different according to Duncan's Multiple range test (DMRT) at 5% level

Table-5 Extraction of bioactive compounds
Preliminary phytochemical screening of the dried callus powder of *Aegle marmelos* with various organic solvents

Medicinal compounds	Petroleum ether	Benzene	Chloroform	Acetone	Methanol
Steroids	+	-	-	+	+
Triterpenoids	-	-	-	-	-
Alkaloids	+	+	+	+	+
Phenolic compounds	-	-	+	+	+
Tannins	-	-	-	-	-
Saponins	-	+	_	+	-
Xanthoproteins	+	-	+	-	+
Flavonoids	+	+	+	+	+
Reducing sugar	-	-	-	-	-

+ - indicates presence
- - indicates absence

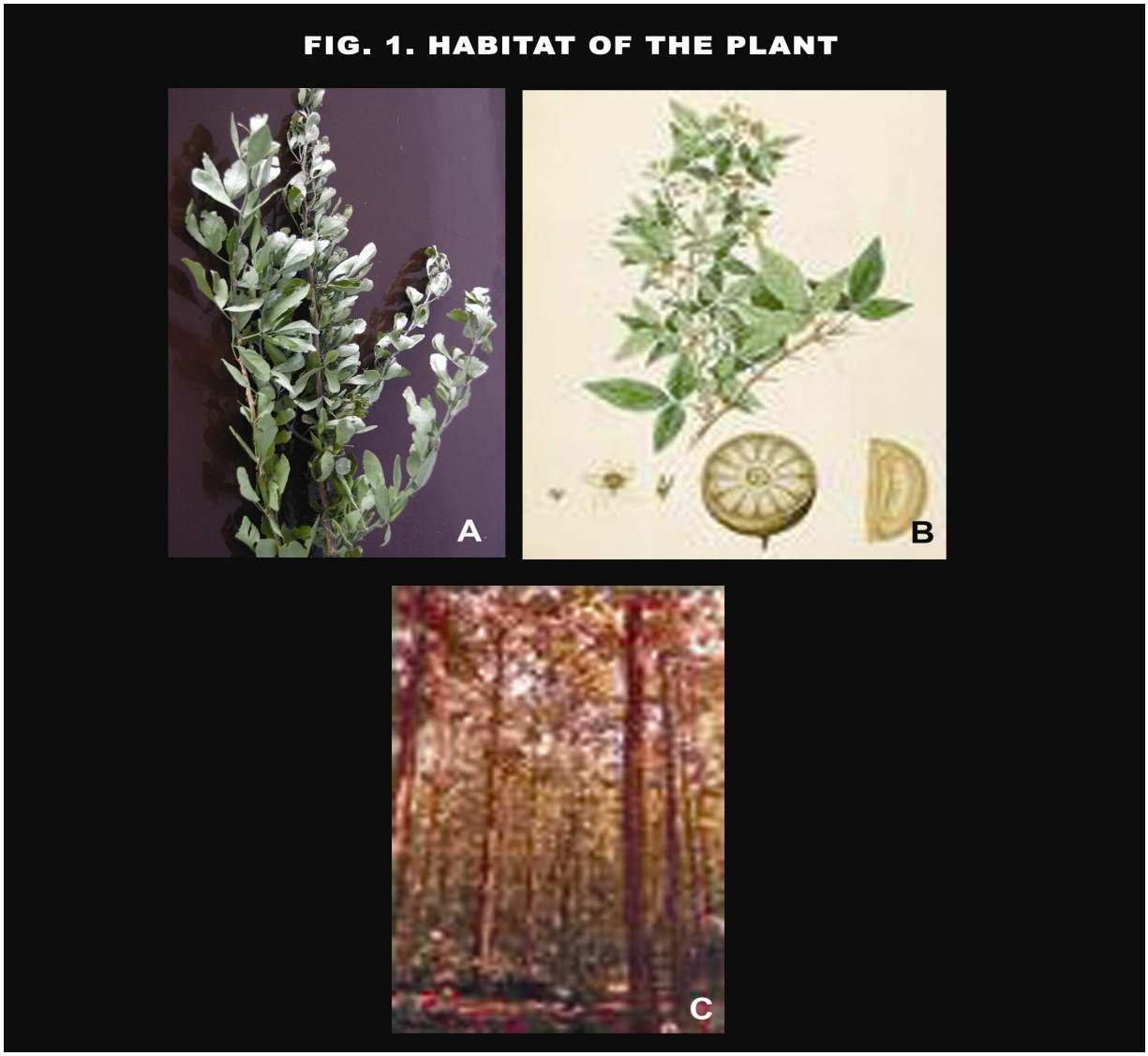

Figure 1. Habit of *Aegle marmelos* (L.) Correa
A-Excised shoots of the plant
B-Figure showing the leaves and fruits of the plant (Net source)
C-The plant in Indo-Burma subcontinent (Net source)

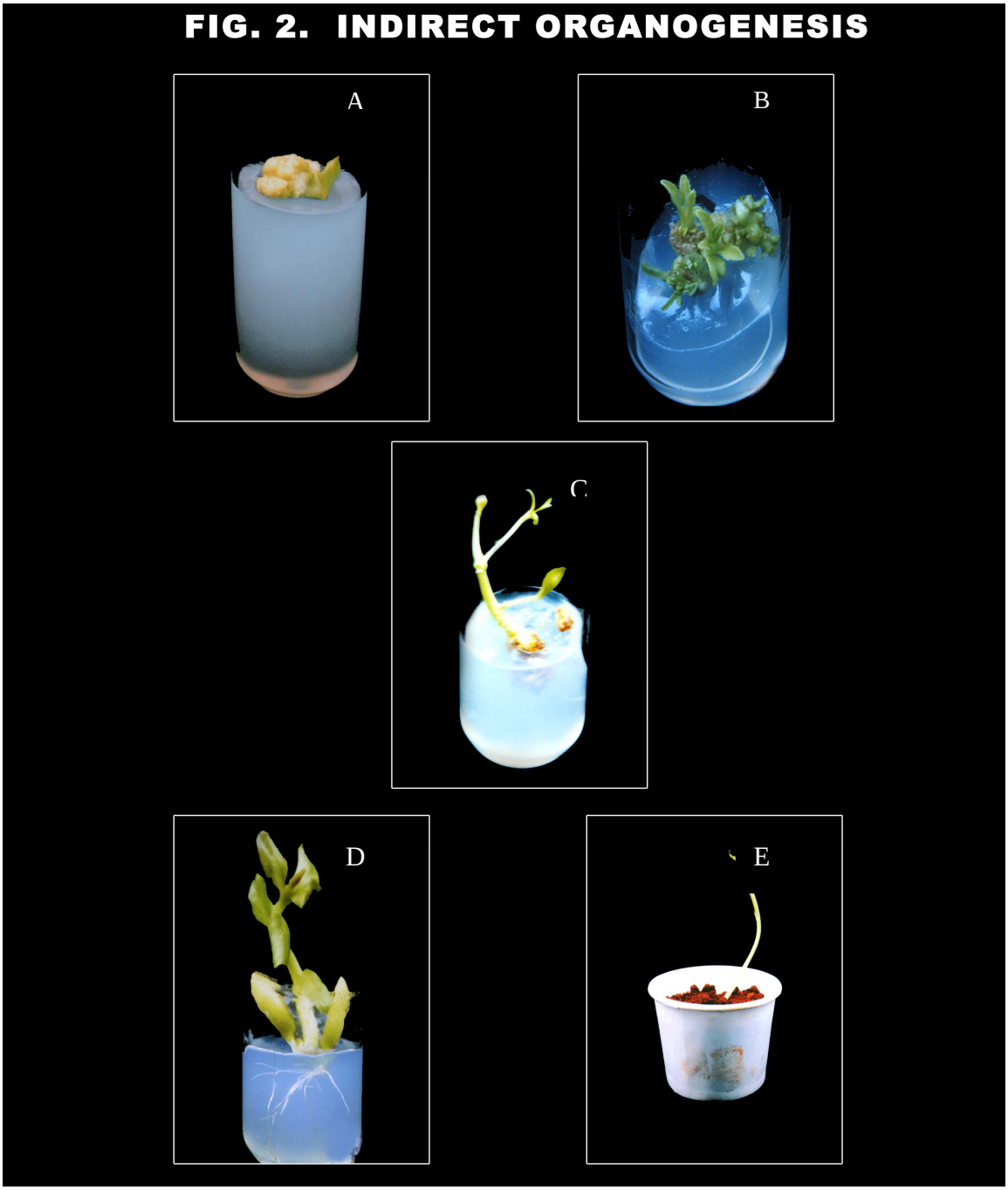

Figure 2. Regeneration of the plantlets from the nodal segments derived callus of *Aegle marmelos* (L.) Correa
A-Greenish, compact and nodular organogenic calli initiating from the nodal explants (MS + 1.5 mg/l 2,4-D + 0.5 mg/l BA)
B-Shoot bud induction (MS + 1.5 mg/l BA + 0.2 mg/l2,4-D)
C-Elongation of the shoots (MS + 0.5 mg/l GA_3 + 1.5 mg/l BA)
D-Rooting on *in vitro* raised shoots (half-strength MS + 1.5 mg/l IBA)
E-Hardened plant

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