SUMMARY
The present study deals with development of *in vitro* propagation protocols and evaluations of genetic fidelity of *Melia dubia* Cav. In this direction, detailed studies conducted are as follows;

I. Axillary shoot proliferation

**Shoot initiation:** effect of type of explant, size of explants, season, plant growth regulators, nutrient media, additives, sucrose, pH, agar-agar concentration and repeated subculturing of differentiated shoot on multiple shoot induction.

**Shoot multiplication:** effect of plant growth regulators, effect of GA$_3$ and adenine sulphate, nutrient media, additives, sucrose, pH, agar-agar concentrations and incubation conditions.

II. Somatic embryogenesis/adventitious regeneration;

Callus induction: Effect of explant types, plant growth regulators, nutrient media, additives and sucrose concentrations.

Callus multiplication: Effect of plant growth regulators, nutrient media, carbohydrate sources and sucrose concentrations.

Callus differentiation: Effect of plant growth regulators

III. Direct adventitious regeneration; Effect of PGRs on direct shoot induction from leaf explant of mature trees (using *in vitro* shoot culture).

IV. *In vitro* rooting; effect of auxins, nutrient media, incubation conditions, and pulse treatment of IBA.

V. *Ex vitro* rooting; effect of pulse treatment of IBA.

VI. Hardening of micropropagated plants

VII. Evaluation of genetic fidelity of micropropagated plants of *M. dubia* through axillary shoot proliferation.

VIII. Evaluation of growth performance of micropropagated plants at nursery stage.
Axillary shoot proliferation

Shoot initiation

Effect of type of explants

Among the apical and nodal shoot segments used as explants for shoot induction in MS medium with additives (ascorbic acid 50 mg/l + citric acid 25 mg/l + cysteine 25 mg/l) + NAA (0.1 mg/l) + BAP (0.5 mg/l), nodal shoot segment exhibited high (88.93%) percent shoot induction in 4 weeks period with sturdy shoot, as compared to apical shoot segment, which produced 70.67% shoot induction at 25 ± 2°C temperature and 37.5 µmol m⁻² s⁻¹ light intensity for 16h photoperiod.

Effect of size of explant

Among the different diameters (5.0-9.0 mm) of the explants (nodal shoot segment) tested for shoot induction in MS medium with additives + NAA (0.1 mg/l) + BAP (0.5 mg/l), maximum (89.50%) frequency of shoot induction was obtained from the explants of 7.0 mm in diameter (medium size). The shoot induction frequency for different diameters of the explants varied from 60.03 to 89.50%.

Effect of season

Effect of season/period of explant collection had significant effect on shoot initiation and it varied from 14.90 to 90.00%. The explants obtained during the month of April exhibited the best response (90.00%) on shoot induction in MS medium, consisted additives + NAA (0.1 mg/l) + BAP (0.5 mg/l) within 4 weeks, followed by the explant collected in the month of March (84.97%).

Effect of PGRs (auxins and cytokinins)

PGRs has shown significant effect on percentage response of shoot induction and it varied from 11.10-90%. Among the various growth regulators; auxins (IAA, NAA and IBA) and cytokinins (BAP, Kn and TDZ) tested in MS medium with additives for shoot initiation. Combined use of BAP (0.5 mg/l) + NAA (0.1 mg/l) in the MS medium, proved to be best for high (90.00) percentage of shoot induction and shoot length (2.80 cm). Higher concentrations of cytokinins (>1.0 mg/l) did not help in multiple shoot induction, but posed problem of callusing from the explant.
Effect of nutrient media

Nutrient media has significant effect on percentage of shoot induction and it varied from 27.66 to 87.43%. Among the different nutrient media (MS, B5, WP, SH, and HE) used with additives + NAA (0.1 mg/l) + BAP (0.5 mg/l), MS medium proved to be the best for maximum (87.43%) response on shoot induction with shoot length 2.63 cm. Minimum (27.66%) shoot induction was observed on SH medium.

Effect of additives

Additives were found to have significant effect on percentage of shoot induction and shoot length. Among the various additives viz; ascorbic acid (50 mg/l), citric acid (25 mg/l), cysteine (25 mg/l), adenine sulphate (25 mg/l), yeast extract (500 mg/l) and casein hydrolysate (500 mg/l) tested in MS medium with NAA (0.1 mg/l) + BAP (0.5 mg/l), medium supplemented with ascorbic acid (50 mg/l) + citric acid (25 mg/l) + cysteine (25 mg/l) proved the best with maximum (89.83%) response on shoot induction with shoot length of 2.37 cm, followed by adenine sulphate (25 mg/l) along with ascorbic acid + citric acid + cysteine, which favored 86.67% of shoot induction with shoot length of 2.10 cm. Percentage shoot induction varied from 55.37 to 89.83% with different additives in the shoot induction medium.

Effect of sucrose concentrations

Various concentrations of sucrose had shown significant effect on percentage response of shoot induction and it varied from 77.47 to 88.59%. Among the different concentrations (1.0 – 5.0%) of sucrose tested in the MS medium with additives + NAA (0.1 mg/l) + BAP (0.5 mg/l), medium with 3.0% sucrose in MS medium proved the best in terms of high (88.59) percentage of shoot induction with maximum shoot length of 2.50 cm, followed by 4.0% sucrose after 4 weeks. Minimum (77.47%) shoot induction from the explant was in the medium with 1.0% sucrose.

Effect of pH

Among the three pH (4.5, 6.0 and 7.0) of the MS medium tested, maximum (90.0%) shoot induction and shoot length (2.6, cm) was observed at pH 6.0 of MS medium consisted additives + NAA (0.1 mg/l) + BAP (0.5 mg/l). No response was
found at lower pH. Minimum (73.34%) shoot induction was observed on the medium with pH 7.0.

**Effect of agar-agar concentrations**

Among the different concentrations of agar-agar (0.4, 0.6, 0.7 and 0.8%) tested in MS medium with additives + NAA (0.1 mg/l) + BAP (0.5 mg/l), medium with 0.7% agar-agar proved to be the best for maximum (89.13) percentage of response on shoot induction with 2.7 cm shoot length in 4 weeks period. Minimum response (68.55%) on shoot induction and growth was found on medium with 0.4% agar-agar concentration.

**Effect of repeated subculturing on multiple shoot induction**

Though, the frequency of shoot initiation was >85% in most of shoot initiation experiments, but produced single shoot. Therefore, to increase shoot number, original explants were sub-cultured along with differentiated shoots on fresh MS medium supplemented with additives + NAA (0.1 mg/l) + BAP (0.5 mg/l) at the interval of every 2 weeks and in 3rd passage cultures, 13.43 shoots/explant were produced with shoot length of 4.57 cm. This method can be used for the production of multiple shoots with good shoot length for further multiplication for large scale production of clonal planting material of *M. dubia*.

**Shoot multiplication**

**Effect of PGRs (auxin and cytokinins)**

Among the various PGRs viz; auxins (NAA and IAA) and cytokinins (Kn, BAP and TDZ) tested either alone or in combinations for shoot multiplication in MS medium with additives has shown significant effect on shoot number and shoot length. BAP (0.5 mg/l) alone in MS medium was found the best cytokinins to maintain high multiplication rate with maximum of 9.83 shoot number per shoot clump (each clump of 2-3 shoots) with mean shoot length 3.73 cm. Shoot clump (2-3 shoots/clump) was found better than single shoot for shoot multiplication in *M. dubia*. It was observed from the results that the use of auxins in the medium with BAP/Kn posed problem of callusing from the shoot base and did not favor shoot multiplication and shoot growth.
Effect of GA₃ and Adenine sulphate

Among the different concentrations of GA₃ (1.5 to 2.5 mg/l) and adenine sulphate (5.0 to 13 mg/l) tested in MS medium with optimum concentration of BAP (0.5 mg/l) for further shoot multiplication and shoot elongation. GA₃ (2.5 mg/l) + AS (5.0 mg/l) in the medium significantly favored maximum production of multiple shoots (14.77±0.64) with maximum shoot length of (5.43±0.06) without callus.

Effect of nutrient media

Nutrient media have shown significant effect on shoot multiplication and growth and it varied from 4.20 to 13.10 number of shoot/clump. Among various nutrient media (MS, B5, WP, SH and HE) tested for shoot multiplication and growth, MS medium with additives + BAP (0.5 mg/l) + GA₃ (2.5 mg/l) + AS (5.0 mg/l) favoured production of highest (13.10) number of shoots per shoot clump with maximum shoot length (4.53 cm). Minimum (4.20) number of shoots/clump was observed on HE medium.

Effect of additives

Additives in the shoot multiplication medium had shown auxillary effect on shoot multiplication and growth. Among the various additives (ascorbic acid, citric acid, cysteine, adenine sulphate, yeast extract and glutamine) tested either alone or in combinations to improve shoot multiplication rate and subsequent shoot growth, addition of adenine sulphate (5.0 mg/l) in medium with ascorbic acid (50 mg/l) + citric acid (25 mg/l) + cysteine (25 mg/l) resulted in highest number (12.50 shoots/clump) shoot production with maximum (5.37 cm) shoot length, followed by 9.20 shoots per shoot clump with 4.80 cm shoot length in MS medium supplemented with ascorbic acid (50 mg/l) + citric acid (25 mg/l) + cysteine (25 mg/l) within 4 weeks.

Effect of different concentrations of sucrose and glucose

Among the sucrose (1.0-4.5 %) and glucose (3.0 %) tested either alone or in combinations as a source of carbohydrates, maximum (18.67 shoot/clump) shoot
multiplication was observed in MS medium consisted 3.0 % sucrose + 1.5% glucose with BAP (0.5 mg/l), but shoot length was significantly less (1.97 cm) as compared to medium with 3.0 % sucrose alone. Whereas, MS medium with 3.0 % sucrose comparatively favored less number of shoots (12.33 shoots) but with maximum shoot length (5.13 cm) which were ideal for further shoot multiplication and rooting.

Effect of pH

Effect of pH of the media was significant on shoot multiplication and it varied from 3.87-13.43 shoots/clump. Among the various pH (4.5- 7.0) of the MS medium tested, maximum shoot multiplication (13.43 shoots/clump) was observed with highest (4.80 cm) shoot length at pH 6.0 of the MS medium with additives + BAP (0.5 mg/l) + GA₃ (2.5 mg/l) + AS (5.0 mg/l).

Effect of agar- agar concentrations

Shoot multiplication varied from 5.73-12.97 shoots per shoot clump with different (0.4-0.8%) concentrations of agar- agar in the medium. Among the various concentrations of agar-agar tested as gelling agent in the MS medium with additives + BAP (0.5 mg/l) + GA₃ (2.5 mg/l) + AS (5.0 mg/l), medium with 0.7% agar-agar proved the best for maximum shoot multiplication (12.97 shoots) with highest (4.50 cm) shoot length. Minimum shoot multiplication was observed on the medium consisted 0.8% agar-agar.

Effect of incubation temperature

Among the various incubation condition (20°C, 25°C, 28°C and 32°C, temperature) tested, shoot multiplication cultures incubated at 25°C temperature favored maximum shoot multiplication (13.17 shoots/clump) with maximum shoot length (5.17 cm) in MS medium with BAP (0.5 mg/l) + GA₃ (2.5 mg/l) + AS (5.0 mg/l), followed by incubation of cultures at 28°C temperature, which produced (10.47 shoots/clump) shoots with 4.47, cm shoots in length. Shoot multiplication varied from 5.38 – 13.17 shoots/clump at different incubation condition. High temperature (32°C) posed problem of shoot drying and callusing from shoot base.
Somatic embryogenesis/adventitious regeneration

Callus induction

Effect of explant

Among the different explants (leaf, internodes, nodal and cotyledon segments) tested in MS medium supplemented with additives (ascorbic acid 50 mg/l + citric acid 25 mg/l + cysteine 25 mg/l + glutamine 100 mg/l) + NAA (0.5 mg/l) + BAP (1.0 mg/l), Leaf segment as an explant proved the best for maximum (100 %) frequency of callus induction with more fresh weight (2.43 g), followed by cotyledon explants (85.12 %) with 2.32 g fresh weight. Minimum (59.33%) callus induction was observed from the internode explant.

Effect of PGRs

Plant growth regulators had shown significant effect on percentage response of callus induction and it varied from 10.33 to 100%. Among the various auxins viz; IAA, NAA and 2,4-D with cytokinins; BAP and Kn tested, MS medium with additives + NAA (0.5 mg/l) + BAP (1.0 mg/l) proved the best for highest response (100%) with fresh weight of 2.44 g, followed by IAA (0.5 mg/l)+ BAP (2.0 mg/l) with 92.50% response with fresh weight of 2.09 g.

Effect of nutrient media

Effect of nutrient media was significant on callusing and it varied from 40.0-100%. Among the various nutrient media viz; MS, B5, WP, SH and HE tested for the callus induction, MS medium supplemented with additives + NAA (0.5 mg/l) + BAP (1.0 mg/l) proved to be the best for high rate (100 %) of callus induction with maximum fresh weight of 2.21g. This was followed by B5 medium (85.0% with fresh weight of 1.43 g) and SH (65.10% fresh weight 0.97 g). Minimum (40.0%) callus induction was observed on HE medium.

Effect of additives

Among the different additives tested in MS medium, combined use of ascorbic acid (50 mg/l), citric acid (25 mg/l), cysteine (25 mg/l) and glutamine (100 mg/l) in
MS medium with NAA (0.5 mg/l) and BAP (1.0 mg/l) proved the best (99.04%) with fresh weight of 2.45g. Callus induction varied from 20.43 to 99.04% with different additives in the medium. Medium without additives induced minimum (20.43%) frequency of callusing.

**Effect of sucrose concentrations**

Effect of sucrose concentrations was significant on frequency of callusing and growth. Among the various concentrations of sucrose (0.0 to 6.0%) tested for callus induction, sucrose 3% in MS medium with NAA (0.5 mg/l) and BAP (1.0 mg/l) + additives proved to be the best on callus induction with highest rate of response (98.66 % with fresh weight of 2.31g), followed by 1.5% (84.67 % with fresh weight of 1.68 g) and 4.5% sucrose (74.83% with fresh weight of 1.33 g).

**Callus multiplication**

**Effect of plant growth regulators**

Among the various concentrations and combinations of PGRs; auxins (2, 4-D, IBA, and NAA) and cytokinins (BAP and Kn) used, MS medium supplemented with additives + NAA, 0.5 mg/l + BAP, 1.0 mg/l favored rapid callus multiplication with fresh weight 5.62g, followed by 2, 4-D, 0.2, mg/l + BAP 2.0, mg/l with fresh weight of 5.28g.

**Effect of nutrient media**

Among the various nutrient media (MS, B5, WP, SH and HE) used for callus multiplication. MS medium with additives + NAA, 0.5 mg/l + BAP, 1.0 mg/l proved the best for callus multiplication with fresh weight (5.43g), followed by 4.11g fresh weight of callus in the WP medium.

**Effect of different carbohydrates sources**

Among the different carbohydrates *viz*; sucrose, glucose, maltose, fructose and dextrose used either alone or in combinations, sucrose (3.0%) in MS medium with additives + NAA (0.5 mg/l) + BAP (1.0 mg/l) proved the best for callus
multiplication with maximum (6.70 g) fresh weight, followed by maltose (3.0%) with callus fresh weight of 5.90g. Minimum callus multiplication was observed on 3.0% fructose with 3.33 g callus fresh weight.

Effect of sucrose concentrations

Among the different concentrations (1.5 to 6.0%) of sucrose used in MS medium with NAA (0.5 mg/l) + BAP (1.0 mg/l), medium with 3.0% sucrose was observed the best concentration for callus multiplication with fresh weight of 6.11g.

Callus differentiation

Effect of plant growth regulators

Out of the various concentration of BAP (0.5-2.5 mg/l) and auxins (1.0-2.0 mg/l) used for callus differentiation in MS medium, Maximum (65.10%) callus differentiation with average 5.30 shoots/callus clump with shoot length of 3.53 cm was observed in MS medium supplemented with additives + BAP (0.5 mg/l) + IAA (2.0 mg/l), followed by 62.33% callus differentiation on MS medium with additives BAP (0.5 mg/l) + IAA (1.0 mg/l). None of the growth hormones treatment induced somatic embryo from the callus.

Direct adventitious shoot regeneration

Effect of cytokinins (Kn and BAP) was found significant on direct adventitious shoot induction which varied from 31.00 to 73.30%. Among the various cytokinins tested for direct adventitious shoot regeneration from the leaf explant, MS medium supplemented with BAP (1.0 mg/l) + Kn (0.1 mg/l) + AS (3.0 mg/l) favored highest (73.30%) percentage of adventitious shoot induction with maximum mean number of shoots (4.19) and shoot length (2.47 cm). Medium with BAP (1.0 mg/l) alone proved least (31.00%) effective in term of adventitious shoot induction.

In vitro rooting

Effect of auxins

Effect of various auxins was significant on rooting percentage from the in vitro shoots and varied from 11.00 -98.00 %. Among the different auxins (IAA, IBA
Summary

& NAA, 0.1-1.5 mg/l) tested in the MS/2 medium for *in vitro* rooting, MS/2 medium supplemented with IBA (0.5 mg/l) was proved the best and favored highest (98.00%) rooting, with more root number (4.33) and maximum root length (4.41 cm). Root induction was not observed on hormone free medium and medium supplemented with IAA and NAA either at lower or higher concentrations favored callus induction from shoot base, and were not found suitable for rooting in *M. dubia*.

**Effect of nutrient media**

Effect of nutrient media was distinct on rooting and it varied from 40.23 to 97.51%. Among the different nutrient media (MS, MS/2, MS/4, WP, HE and White’s) tested with IBA (0.5 mg/l), MS/2 medium was found best in terms of the frequency of rooting (97.51%), number of roots (4.57) and root length (4.27, cm). Minimum (55.33%) rooting frequency was observed on MS medium.

**Effect of incubation temperature**

Among the various incubation conditions viz; 20º C to 32º C temperature tested for rooting in MS/2 medium with IBA (0.5 mg/l), maximum (97.41%) root induction, root number (3.67) and maximum (4.93 cm) root length was observed at 25ºC temperature, followed by at 28ºC temperature, which favored 60.58% root induction, but shoots were gradually dried within four weeks possibly due to callusing at the base. Incubation temperature of 32º C was not found suitable for rooting and shoots dried within 3-4 weeks period.

**Effect of pulse treatment of IBA**

Rooting percentage varied from (15.46 to 90.35%), with different concentrations of IBA (250-1000 ppm) used as pulse treatment of shoots for different duration (15 and 30 minutes) and subsequently, transfer of such IBA treated shoots to hormone free half strength MS medium. Micro shoots pulse treated with IBA (250 ppm) for 15 minutes, followed by transfer of shoots on hormone free MS/2 basal medium resulted in maximum (90.35%) root induction with highest (4.87) number of roots and maximum root length (4.72 cm). Minimum (15.46%) rooting frequency was observed in shoots treated with 1000 ppm IBA for 30 min and followed by transfer of shoots to MS/2 medium.
Ex vitro rooting

Effect of pulse treatment of IBA

Among the various concentrations (250-1000 ppm) of IBA used for pulse treatment for 15 to 30 minutes and transferred such pulse treated shoots in to autoclaved soilrite for ex vitro root induction, maximum (50.27%) percentage rooting with (2.67) maximum number of root and root length (1.07 cm) was observed from the shoots treated with IBA (500 ppm) for 15 min. Shoots were completely dried, which pulse treated with IBA for longer duration.

Hardening of micropropagated plants

Hardening of rooted shoots was found essential for 4 weeks in polytunnel in green house and four weeks inside the green house without polytunnel, before keeping in shade house for one month to obtain high rate (> 95%) of survival of the micropropagated plants.

Evaluation of genetic fidelity of micropropagated plants

RAPD markers

Out of the 20 decamer RAPD primers screened, only 11 decamer oligonucleotides produced good amplification products in term of quality and quantity of banding patterns, while the rest of the primers resulted in either no amplification or smeared profiles. The number of bands produced by a single primer ranged from 3 (OPQ-15) to 11 (OPA-09). Total 72 bands were produced with an average 6.55 bands per primer. The bands obtained using all 11 primers were found to be monomorphic across all the micropropagated plants from one year old shoot multiplication cultures and RAPD profile revealed no variation in micropropagated plants and they were found genetically stable.

ISSR markers

In case of ISSR fingerprinting, 20 anchored microsatellite primers including di-, tri-, and tetra nucleotide repeat motifs were individually tested to amplify DNA
from the mother plant and 10 randomly selected micropropagated plants. Out of which, 10 primers generated well resolved reproducible banding profile. The total 80 scorable bands ranged in size from 250 bp to 4000 bp and produced with an average of 8 bands per primer. The amplified bands were maximum (11) in Primer UBC-836 and minimum (4) in primer number UBC-855. The banding patterns of amplified products were found to be monomorphic across all the progenies and no variation was detected in micropropagated plants.

**Evaluation of growth performance of micro propagated plants at nursery stage**

Hardened plants transferred to open nursery and evaluated for growth performance at nursery stage up to ten month at the interval of two months. Steady increase in the height and collar diameter was observed. Ten month old plants attained height of 109.48±1.56 cm with 40.07±0.60 mm collar diameter.

**Significant Findings of the study**

**Axillary shoot proliferation**

**Shoot initiation:**

- Nodal shoot segment of 7.0 mm diameter, collected during the month of April used as an explant in MS agar gelled (0.7%) medium with additives (ascorbic acid 50 mg/l, citric acid 25 mg/l and cysteine 25 mg/l), NAA (0.1 mg/l), BAP (0.5 mg/l), sucrose, (3.0%) and pH of 6.0, proved the best for high (90.00%) frequency of shoot induction in *M. dubia*.

- Multiple shoots (13.43) could be achieved in 10 weeks by subculturing of explant along with differentiated shoots for 3 passages at the interval of 2 weeks on the fresh MS medium with additives, NAA (0.1 mg/l), BAP (0.5 mg/l).

**Shoot multiplication:**

- MS medium with 3% sucrose, additives (ascorbic acid 50 mg/l, citric acid 25 mg/l and cysteine 25 mg/l), NAA (0.1 mg/l), BAP (0.5 mg/l), GA$_3$ (2.5 mg/l),
AS (5.0 mg/l) and solidified with 0.7% agar-agar proved the best in terms of maximum shoots (14.77±0.64 shoots/clump) with shoot length (1.95 cm) in 3 weeks period at 25°C temperature and 2500 lux intensity of light for 16 h photoperiod.

**Somatic embryogenesis/adventitious regeneration**

Adventitious shoot induction was achieved either through callus phase or by direct adventitious regeneration.

- MS medium with additives (ascorbic acid 50 mg/l, citric acid 25 mg/l, cysteine 25 mg/l and glutamine 100 mg/l), NAA (0.5 mg/l) and BAP (1.0 mg/l) proved the best for callus induction and multiplication from leaf segments.

- MS medium with additives (ascorbic acid 50 mg/l, citric acid 25 mg/l, cysteine 25 mg/l and glutamine 100 mg/l), IAA (2.0 mg/l) and BAP (0.5 mg/l) proved the best for adventitious shoot induction from callus.

- Direct adventitious shoot regeneration can be obtained on MS medium with additives (ascorbic acid 50 mg/l, citric acid 25 mg/l, cysteine 25 mg/l and glutamine 100 mg/l), BAP (1.0 mg/l), Kn (0.1 mg/l) and AS (3.0 mg/l) from leaf explant under dark for 4 weeks followed by light at 25±2°C temperature.

**In vitro rooting**

- MS/2 medium supplemented with IBA (0.5 mg/l) proved the best and favored highest (98.00%) rooting without callus induction at base of shoots, with mean number (4.33) number of roots and maximum root length (4.41, cm).

**Hardening of micro propagated plants**

- *In vitro* rooted plants were successfully hardened in the greenhouse within 8 weeks period and survival rate was found more than 95%.
Genetic fidelity studies

- Genetic fidelity studies through RAPD and ISSR marker revealed no variations among micropropagated plants raised from one year old shoot multiplication cultures and they were true to type to mother plants.

Growth performance of micro propagated plants at nursery stage

- At the age of ten months, micropropagated plants attained height of 109.48±1.56 cm with 40.07±0.60 mm collar diameter.