J. Phytol. Res. **35**(1): 1-10, 2022 **ISSN 0970-5767**

IMPROVISED CLONING PROTOCOL WITH CONCURRENT *EX VITRO* RHIZOGENESIS AND GENETIC HOMOGENEITY PROBING OF *ZIZYPHUS MAURITIANA* LAM.

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Zizyphus mauritiana is a medicinally important plant species of arid ecosystem. High degree of heterozygosity, over exploitation and low multiplication rate are major constraints in propagation of this plant species. Here we report a cost- effective cloning protocol of *Z. mauritiana*. Murashige and Skoog (1962) medium with BAP (2.0 mg l^{-1}) + additives found suitable for axillary shoot induction from mature nodal explant. On this medium 2.8 ± 0.67 shoot were produced from each axillary meristem. Shoots were further multiplied by sub-culturing in MMS medium containing various concentrations of plant growth regulators (BAP, Kn, 2iP) in different combinations. The *in vitro* propagated shoots were treated with IBA or/and NOA for *ex vitro* rhizogenesis. *Ex vitro* rooted plantlets were transplanted to the soil with 95% rate of survival. These plantlets were tested for genetic homogeneity using SCoT marker. For the tested primers, non-detectable variation was observed in DNA profiling among the micropropagated plants and the mother plant. To the best of our knowledge, this is the first report on concurrent *ex vitro* rhizogenesis with acclimatization and SCoT based genetic homogeneity assessment of *Z. mauritiana*. Optimal multiplication and higher survival rate coupled with clonal stability, ensures the effectiveness of the protocol.

Keywords: Cloning, *Ex vitro* rooting, Genetic Homogeneity, *In vitro* - Shoot multiplication, SCoT

Introduction

Zizyphus mauritiana Lam. (Rhamnaceae) is native to areas from India to South-Western China, Malaysia and is cultivated over vast areas of Asia[1]. The fruits (known as 'Indian jujube' in English, 'Ber' in Hindi) possess sweet tasting pulp and source of antioxidants like ascorbic acid, carotenoids, and vitamin C. Its vitamin C content is found to be higher than citrus fruits such as oranges. The fruits are used as a folk medicine for anorexia, dyspepsia, leprosy, nausea, pitta, pruritis, and ulcers. Leaves are rich sources of iron, magnesium, zinc, and calcium used to treat diarrhea, fever, gastric disorder, liver damage, and pulmonary disorders[2 & 3].

Continuous exploitation due to increasing demand, without replenishment and restoration, has resulted in a decrease population of this tree species. *Z .mauritiana* is naturally propagated through seeds, but this method is not suitable for elite genotype propagation. Because seeds have a high degree of heterozygosity[4] and the multiplication rate is low. Therefore, to meet the increasing demand for this plant

material, *in vitro* cloning is a viable method. *In vitro* regeneration method through shoot tip culture and nodal shoot segments in *Z. mauritiana* have been reported earlier[4-7]. However, the rate of multiplication and survival was not so good in these reports. Further, *ex vitro* rhizogenesis and genomic uniformity/true-to-type nature of the regenerated plants have not been reported earlier. *Ex vitro* rhizogenesis of micro-propagated plants is advantageous because it does not require an additional step of acclimatization[8] and reduces the cost, labor, and time[9]. It is also crucial to evaluate the genetic stability of the regenerants for true-to-type cloning of elite genotype[10]. PCR-based molecular markers, i.e., SCoT, ISSR, SSR, and RAPD, are being successfully employed to monitor the genomic homogeneity in tissue culture raised plantlets of several species[11-13]. The present study was undertaken to (1) evaluate the effect of plant growth regulators on *ex vitro* rhizogenesis along with improvisation of *in vitro* morphogenesis protocol and to (2) assess the genetic stability of the regenerants using SCoT and RAPD markers.

Material and Methods

In vitro morphogenesis:

For *in vitro* morphogenesis, a healthy and mature tree with better fruit yield was selected as a mother plant. Nodal segments (2-3 cm long) were harvested from July to September and used as explants. The treated explants were inoculated in a sterile MS nutrient medium [14] with agar (0.8%) and 3% sucrose, additives[15] and PGR's. Different concentrations (1.0, 2.0, 3.0 mgl^{-1}) of PGR's (BAP / Kn) were tested. PGR free medium was treated as control. Culture conditions remain the same as described previously for *Terminalia arjuna*[16]. Axillary shoots produced *in vitro* were harvested and cut into 3–4 cm segments containing 1–2 nodes. They were sub-cultured in fresh MMS medium supplemented with additives, AC (100 mg l^{-1}), and PGR's. Different cytokinins (BAP, 2iP, Kn) at different strength (0.25, 0.5, 1.0 mg l^{-1}) were evaluated. Cultures were proliferated under a light intensity of 40–50 µmol m^{-2} s^{-1} PFD for 12 h day^{-1}, temperature (26 ± 2 °C) and RH (60 %) with regular sub-culturing after 15-17 days.

Ex vitro rhizogenesis, acclimatization and hardening:

Harvested *in vitro* shoots were cleaned with tap water and pulse-treated with root-inducing PGR's. For this, the basal end (3–5 mm) of excised shoots was submerged in auxin solution for 4 min. Different auxin (IBA, NOA, and combination of IBA + NOA) solutions at different concentrations (0 – 500 mg l^{-1}) was evaluated to optimize for *ex vitro rhizogenesis*. Treated shoots were transferred to bottles containing sterile Soilrite® (Keltech Energies Ltd., India) and nourished with one-fourth strength of MS medium. These bottles were gradually shifted from the pad section (28±2°C and RH 80–90%) to the fan section of the green house during the process of acclimatization. The number and length of the roots were noted down after 12-15 days. For hardening of the plantlets, caps of bottles were unscrewed progressively after18-20 days, and the bottles were gradually shifted towards the fan section (32±2°C and RH 50–60%). After 5–6 weeks, *ex vitro* rooted plantlets were transferred to a combination of soil and manure (1:1) in poly bag. After 2 months, these hardened plantlets were transferred to the garden pot containing field soil in the nursery.

Genetic homogeneity probing:

Genetic homogeneity was evaluated using SCoT and RAPD markers. Healthy leaves (100 mg) of seven *in vitro* regenerated six-month-old plants and mother plant were collected and fixed using liquid nitrogen.DNA was isolated and purified using modified CTAB method[17]. Quality and quantity of DNA were checked using 0.8% (w/v) agarose gel electrophoresis (AE-

6125/6133 ATTO, Japan) and UV–Vis Spectrophotometer (Elico). After preliminary screening, 15 SCoT primers were selected[18]. RAPD analysis was done using 10 RAPD decamer of operon series (Table 3).The reaction mixture for both the markers and amplification conditions for RAPD are described previously. The thermal cycler program for SCoT markers is set as mentioned in *Alhagi maurorum* [16]. The PCR products were resolved and data were evaluated as described by Harish et al.[19]

Experimental design and data analysis:

Experiments were performed thrice and twenty explants were used in each experiment. Results are expressed as mean ± SD and raw data are subjected to one-way analysis of variance (ANOVA) at $P< 0.05$ level of significance using SPSS v.17 (SPSS, Chicago, USA). Grouping of means is done using Duncan's multiple range test (DMRT).

Results and Discussion

In vitro morphogenesis:

Induction of axillary shoot bud was observed after 12-15 days of explant inoculation. MS medium without cytokinin did not prop-up axillary bud induction and eventually, necrosis occurs in the explant. Among the cytokinins tested, BAP was most suitable for axillary shoot bud induction (Fig. 1A, 1B). The superior impact of BAP over Kn is well documented in many plant species[20-21]. Further, nutrient medium containing additives proved better. Among additives used, adenine sulphate acts as supplementary cytokinin and improves morphogenesis. It has been reported that incorporation of adenine sulphate in culture medium improves the shoot multiplication in *Withania somnifera*[22]. Ascorbic acid and citric acid act as a source of antioxidants, while L-arginine provides reduced nitrogen to developing cultures[23]. Beneficial effects of these additives on *in vitro* morphogenesis have been reported in *Terminalia arjuna*[16] and *Mitragyna parvifolia*[8]. *In vitro* raised shoots were sub-cultured in MMS medium supplemented with different concentrations and combinations of cytokinins. The MMS medium fortified with BAP (0.25 mg l^{-1}) + Kn (0.25 mg l^{-1}) + 2iP (0.25 mg l^{-1}) and additives proved most effective for shoot proliferation. On this medium, 6.53 ± 0.51 shoot of 4.73±0.26 cm length was produced (Table 1, Fig. 1C). This optimum shoot multiplication could be due to a lower concentration of nitrogen salts, incorporation of additives, and multiple cytokinins in medium [22& 24]. In earlier reported protocol for *in vitro* morphogenesis of *Z. mauritiana*, a very high concentration (7.5 mgl^{-1}) of cytokinin (BAP) is used and that method regenerated 4-5 shoot number from nodal explant[7], while present report improvises the previous protocol by using lower concentration of cytokinins (BAP; 0.25 mg l^{-1} + Kn; 0.25 mg l^{-1} + 2iP; 0.25 mg l^{-1}) with higher number of shoot numbers. Further, this improvised protocol uses MMS medium, which has half of the concentration of nitrates. Therefore, the present report resulted in an overall cost reduction with a higher multiplication rate of *Z. mauritiana*.

Further previously, four axillary shoots have been induced in MS medium containing 1.0 mgl^{-1} of BAP and that too with only 65% final plant survival rate[25], however, in the present investigation, we have reported more numbers (>6) of axillary shoot induction with much better survival rate (95%; discussed later) of the regenerates. Chhajer and Kalia[26] also reported that using a combination of more than one cytokinin is more beneficial than using only a single type of cytokinin. Problems like browning of medium and shoot tip burning during multiplication are successfully overcome by supplementation of culture media with AC (100 mg l^{-1}). The use of AC has been found advantageous in other reports as well [27-29].

Ex vitro rhizogenesis and hardening:

Root induction from *in vitro* raised shoots is a crucial stage, and it becomes a challenging

factor when mature trees are concern. In the present study, we developed the *ex vitro* rooting method for *Z. mauritiana*. Table 2 shows the effect of pulse treatment of auxin (both types and concentration) on root

Table 1: Effect of plant growth regulators on shoot proliferation in *Z. mauritiana.*

BAP (mgl^{-1})	Kn (mgl^{-1})	2iP (mgl^{-1})	% Response (Mean±SD)	Shoot Number (Mean±SD)	Shoot Length(cm) (Mean±SD)
0.0	0.0	0.0	00.0 ± 0.00^{e}	0.00 ± 0.0^{d}	0.00 ± 0.00^{f}
0.5	0.0	0.0	40.5 ± 4.37^{b}	1.46 ± 0.51^{b}	2.02 ± 0.26^{e}
1.0	0.0	0.0	32.0 ± 4.21^{c}	1.20 ± 0.41^{c}	2.68 ± 0.27^{b}
0.0	0.5	0.0	26.0 ± 5.16^{c}	1.20 ± 0.14^{c}	2.19 ± 0.48^{d}
0.0	1.0	0.0	13.0 ± 4.83^{d}	1.10 ± 0.30^{c}	2.42 ± 0.51^{c}
0.0	0.0	0.5	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
0.0	0.0	1.0	0.00 ± 0.00^{e}	0.00 ± 0.00^{d}	0.00 ± 0.00^{f}
0.5	0.5	0.5	53.0 ± 5.86^{b}	1.73 ± 0.59^{b}	2.56 ± 0.33^{b}
0.25	0.25	025	87.5 ± 6.34^{a}	6.53 ± 0.51^{a}	4.73 ± 0.26^{a}

Medium: MMS + additives + AC
Data was recorded after 15-17 days of inoculation.
Data having the identical letter in a column was not significantly differed by DMRT ($P < 0.05$).

Table 2: Effect of plant growth regulators on *Ex Vitro* Rhizogenesis in *Z. mauritiana.*

IBA (mgl^{-1})	NOA (mgl^{-1})	Percent Response% (Mean±SD)	Root Number (Mean±SD)	Root Length(cm) (Mean±SD)
0.0	0.0	0.00 ± 0.0^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
100	0.0	0.00 ± 0.0^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
250	0	45.00 ± 5.0^{b}	1.93 ± 0.70^{b}	1.86 ± 0.41^{b}
0.0	100	00.00 ± 0.0^{c}	0.00 ± 0.00^{c}	0.00 ± 0.00^{c}
0.0	250	36.66 ± 5.77^{b}	1.60 ± 0.50^{b}	1.29 ± 0.35^{b}
500	250	91.60 ± 2.88^{a}	5.13 ± 0.63^{a}	3.24 ± 0.30^{a}

Data was noted after 12-15 days of inoculation.
Data having the identical letter in a column was not significantly differed by DMRT ($P < 0.05$).

development. On IBA treatment (250mg l^{-1}) 45±5.0% response with 1.93±0.70 root number and 1.86±0.41cm root length was observed. The advantageous effect of IBA over other auxins like IAA, NOA, etc., on rhizogenesis, has been reported in different plants[30]. This is due to its preferential uptake, transport and metabolism. Besides this, IBA enhances the internal free IAA, or it may act to synergistically alter the synthesis of IAA within plants[31]. The best response (91.60 ± 2.88 %) of rooting was observed when *in vitro* regenerated shoots were pulse-treated with a mixture of IBA (500 mg l^{-1}) + NOA (250 mg l^{-1}). On this combination, 5.13±0.63 roots per shoot with 3.24±0.3 cm root length were regenerated within 12-15 days (Fig. 1D; Table 2). The

beneficiary effect of more than one auxin combination like IBA and NOA on *ex vitro* rhizogenesis has been testified in *Terminalia arjuna*[16].

Table 3: Amplicons number and size (range) generated by SCoT and RAPD marker analysis in *Z. mauritiana*

Primer code	Primer sequence (5'→ 3')	No. of Amplicons	Size of Amplicons (bps)
SCoT			
S-1	CAACAATGGCTACCACCA	4	300-900
S-2	CAACAATGGCTACCACCC	2	200-900
S-3	CAACAATGGCTACCACCG	4	200-800
S-4	CAACAATGGCTACCACCT	7	100-900
S-5	CAACAATGGCTACCACGA	4	200-900
S-6	CAACAATGGCTACCACGC	3	100-900
S-7	CAACAATGGCTACCACGG	4	300-800
S-8	CAACAATGGCTACCACGT	3	300-800
S-13	ACGACATGGCGACCATCG	4	200-800
S-16	ACCATGGCTACCACCGAC	8	200-800
S-18	ACCATGGCTACCACCGCC	5	300-900
S-19	ACCATGGCTACCACCGGC	4	300-800
S-21	ACCATGGCTACCACCGGG	2	200-900
S-28	CCATGGCTACCACCGCCA	3	100-800
S-34	ACCATGGCTACCACCGCA	3	300-700
RAPD			
OPG-03	GAGCCCTCCA	3	300-800
OPH-05	AGTCGTCCCC	4	300-900
OPA-13	CAGCACCCAC	4	300-800
OPAB-18	CTGGCGTGTC	3	400-900
OPB-07	GGTGACGCAG	4	300-900
OPC-02	GTGAGGCGTC	4	300-900
OPBC-09	GTCATGCGAC	2	400-700
OPA-12	TCGGCGATAG	3	300-700
OPH-07	CTGCATCGTG	2	400-600
OPB-01	GTTTCGCTCC	5	300-900

It is important to point out that *ex vitro* rooting is advantageous over *in vitro* rooting due to being cost-effective (reduce up to 30 – 70% cost), time-efficient, have more ecological and physiological vigor to cope up with the stresses faced during hardening. Further, root develops through *ex vitro* rhizogenesis does not require additional steps of acclimatization, which is otherwise done before transplanting of *in vitro* raised plantlets to the natural environmental conditions[8, 32 & 33]. Although *in vitro* rooting protocol has been developed earlier[6-7 & 22], this communication is novel in the sense that *ex vitro* rhizogenesis and the acclimatization in *Z. mauritiana* is reported for the first time. Plantlets rooted *ex vitro* were acclimatized and grew efficiently in a greenhouse environment (Fig. 1E). After 35–40 days of acclimatization, plantlets (8–10 cm) with 6–8 leaves were relocated to poly bags. After 50-60 days of hardening under greenhouse conditions, the plantlets were transferred to garden pots and then shifted to the nursery (Fig. 1F). About 95% of plantlets survived successfully. This percentage of survival was very high in comparison to that of previous reports. This could be due to

adventitious root formation during *ex vitro* rhizogenesis, which are similar to the tap root systems (Fig. 1D), thereby increasing the chance of survival of *in vitro* raised plantlets [33 & 36].

Figure 1: *In vitro* propagation of *Z. mauritiana* **A**. Axillary shoots bud induction on MS containing BAP (2.0 mg l^{-1}) **B**. Repeated transfer on MMS medium containing BAP (0.5mg l^{-1}) and Kn (0.5 mg l^{-1}) **C**. Shoot multiplication on MMS medium containing BAP (0.25 mgl^{-1}) + Kn (0.25 mg l^{-1}) + 2iP (0.25 mg l^{-1}) + AC (0.1%) **D.** *Ex vitro* rooted shoot after treatment with IBA (500 mgl^{-1}) and NOA (250 mgl^{-1}) **E**. Hardening of micro-propagated plants in the greenhouse **F.** Hardened plant of *Z. mauritiana* in garden Pots

particularly when the aim is for large-scale propagation of elite genotype. In the present study, 15 SCoT primers produced a total of 60 bands, which are clearly visible, and gave consistent amplification. The average number of bands per primer was 4.0, with sizes ranging from 100 to 900 bp lengths (Table 3). The number of bands for each

Genetic homogeneity probing:

Evaluation of genetic fidelity in tissue culture-raised plantlets is crucial, primer ranges from two (SCoT-2) to eight (SCoT-16). Fig. 2A shows the SCoT amplification pattern obtained with SCoT-16primer. We also used RAPD marker to support the results of SCoT markers (Fig.

2B). All the RAPD markers resulted in a total of 34 bands, with an average of 3.4 bands per marker and size ranging between

300 to 900 bp sizes (Table 3). The amplification products found monomorphic across all seven micro-propagated plantlets and mother plant, confirming the genetic homogeneity of *in vitro* raised plants (Fig. 2A, 2B; Table 3). The use of SCoT and RAPD marker in genetic fidelity and diversity analysis has been successfully demonstrated in various plant species [16, 34-35]. There are no previous studies on assessing

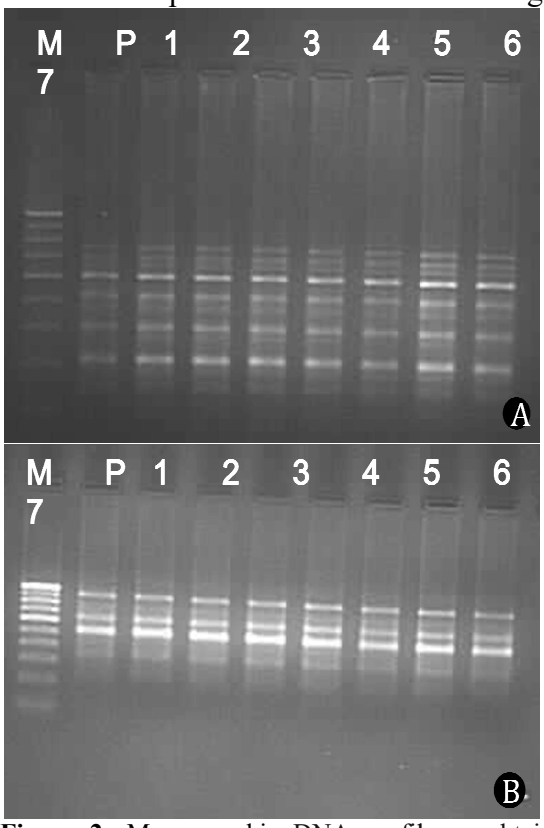

Figure 2: Monomorphic DNA profile as obtained with SCoTand RAPD markers **A.** SCoT primer (S-16) **B.** RAPD primer (OPB-07) M-100 bp Ladder, P - Mother plant, 1-7 - *In vitro* raised plants

the genetic homogeneity of *in vitro* raised plantlets of *Z. mauritiana*.

Conclusion:

In this communication, an efficient *in vitro* morphogenesis protocol is reported with a high multiplication rate. Further, a novel *ex vitro* rhizogenesisis done for the first time in *Z. mauritiana*. This method is found free from soma-clonal variations, as revealed by genetic homogeneity investigation of *in vitro* raised plantlets using two PCR-based markers. Therefore, the protocol is found suitable for mass propagation and cloning of the elite genotype of Z. *mauritiana*.

Declaration of competing interest

The authors declare no conflicts of interest.

Acknowledgment

We acknowledge the financial support from Council of Scientific and Industrial Research (CSIR), New Delhi, to the first author TA. We are thankful to Department of Biotechnology, Government of India, New Delhi, for establishing Micropropagation and Hardening Unit at Jai Narain Vyas University, Jodhpur, Rajasthan (India). We are also thankful to UGC, New Delhi for sanctioned the BSR Start up Grant to author AKG.

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