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Micropropagation of *Jatropha maheshwarii* Subr. & Nayar, an endemic species restricted to the southern terminus of India

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Abstract
An efficient micro propagation protocol for mass multiplication of *Jatropha maheshwarii*, a traditionally important endemic species is described. Explants were collected from its natural habitat to derive plantlets through *In vitro* technique. Morphogenetic potential of somatic explants like shoot tip, axillary bud and leaf discs were studied by culturing them on Murashige and Skoog's medium, either alone or in combinations, with various plant growth regulators namely benzyl amino purine, indole-3-acetic acid, naphthalene acetic acid, kinetin and 2, 4-dichlorophenoxyaceticacid. Inoculated shoot tips expressed 90% shoot growth with BAP+IAA (1mg/l each) treatment. However, with axillary bud explants BAP in combination with KIN (1mg/l and 0.5mg/l) showed profuse shoot budding as well as multiple shoot formation in a faster pace at 95% response rate. Inoculated leaf disc explants showed callus proliferation all over the surface of explants on medium augmented with 2, 4-D (2.0 mg/l). Further caulogenesis and rhizogenesis were also noticed in about 45 days. The shoots rose from shoot tip and axillary bud explants were transferred to rooting medium containing NAA and IBA, to enhance root formation. Where, IBA (1mg/l) seems to be an effective hormone for rhizogenesis with 90% rooting response. The plantlets regenerated were hardened efficiently and transferred to the field successfully with 80% survival rate. This protocol can be used for the mass propagation of the species and thereby conserving its valuable genetic resource.

Keywords: *Jatropha maheshwarii*, Micro propagation, MS Medium, Shoot tips, Axillary buds, Leaf discs.

1. Introduction
Almost all pharmaceutical companies of the globe vastly depend on natural medicinal plant populations for its raw materials. Because of continuous over-exploitation, urbanization, habitat destruction and climatic changes, the status of majority of the medicinal flora is getting endangered in a faster pace. Hence there is a dare need for conservation as well as sustainable utilization of these valuable plant resources for the future generation. The micro propagation technique paves way for enormous production of plants at commercial scale as well as in a means of conservation. Now a days this technique practically plays a major role in the conservation of a wide range of rare (Holobiuc *et al.*, 2009) [8], threatened (Pence, 2005) [18], endemic (Nasircilar *et al*., 2011) [14], endangered (Uzun *et al*., 2014) [27] and recalcitrant species (Sarasan *et al.*, 2006) [21]. The protocols derived through micropropagation will be worthful and useful when classical propagation methods for a species pose negative responses (Fay, 1992 [7], Wala and Jasrai, 2003 [30], Panayotova *et al.*, 2008 [15]).

The genus *Jatropha* of spurge family is morphologically diverse encompassing more economically valuable species. It is distributed in India with 13 species. Among, *Jatropha maheshwarii* Subr. and Nayar is an endemic species distributed in the southern coastal belts, plains and hilly regions of Kanyakumari, Thoothukudi and Tirunelveli districts of Tamil Nadu, extending to the west coast up to Thiruvananthapuram district of Kerala (Abdul Kader, 2014) [1]. This plant is known locally in Tamil as 'Athalai' (Ahmedullah and Nayar, 1986) [2], 'Vel-athalai' (Ben *et al*., 2014) [4] and 'Kattamannaku' (Maria Sumathi and Uthayakumari, 2014) [12]. It is an evergreen rhizomatous under shrub which attains a height of about 2m having 22 chromosomes (Parthiban *et al*., 2009) [17]. This plant is notable among the locals for its valuable traditional medicinal properties against rheumatism, eczema, ringworms and as an insecticide (Maria Sumathi and Uthayakumari, 2014 [12]; Uthayakumari and Sumathy, 2011 [26]). The latex obtained from the plant parts is reported to have potential to arrest hemorrhage from eczema and also to treat mouth ulcers. The leaf extract is reported to treat inflammations and possess anti-inflammatory activity (Ben *et al*., 2014) [4]. Fresh tender stems are utilized as tooth brush by the local community. Further antimicrobial efficacy of stem extract (Viswanathan *et al*., 2004) [28], plant extract (Ben *et al*., 2014) [4], rhizome and fruit pulp extracts (Ben and Sivanadanam, 2015) [5] as well as the antioxidant activity of tuber extracts were also reported (Sakthidevi and Mohan, 2014) [20].

Seeds produced by this species show a very low percentage of germination due to barriers like non-viability, impotency etc. Macropropagation methods like rooting of branch cuttings were less successful. Therefore the propagation and conservation of this species is an impending need. So far, no effort has been made for *in-vitro* propagation of this species. Hence, an attempt has been made for developing an efficient

protocol for *in-vitro* propagation and mass production of this endemic species.

2. Materials and methods

Fresh disease free shoots and branches of *J. maheshwarii* were identified from the shore areas of Kanyakumari coast and were collected in polythene bags, wrapped with moist cotton to prevent desiccation. These shoots were brought to the laboratory which served as the source of explants.

Healthy and young explants like shoot tips, axillary buds and leaf discs were collected. The excised explants were thoroughly washed in running tap water. Surface contaminants were removed by transferring the explants into water containing 1-3 drops of surfactant (Teepol) for 10 – 20 minutes followed by 70 percent ethyl alcohol for three seconds and then with 0.5 percent sodium hypochlorite solution for 3 – 5 minutes. The explants were again washed with double distilled sterile water for at least seven times. Finally these somatic explants were trimmed to 0.5 to 1 cm length and were inoculated aseptically on culture vials containing MS medium (Murashige and Skoog, 1962) [13] with 3 percent sucrose and various PGR's. The pH of the medium was adjusted to 5.8 before adding 0.8 percent agar. The culture medium was autoclaved at 121°C for about 15 minutes. The inoculated culture vials were incubated at 24 ± 2°C under cool fluorescent white light (4000 lux, 16hr/d) and with 60 – 65 percent relative humidity. All experiments were repeated thrice.

Shoot tips and axillary buds were cultured on MS medium fortified with cytokinin (BAP) and auxins (IAA, NAA, KIN and 2, 4-D) for direct shoot and callus induction. Shoot buds were regenerated directly from shoot tip and nodal explants which were transferred to the rooting medium fortified with NAA and IBA for rhizogenesis. Leaf disc explants were cultured on the medium supplemented with 2,4-D and BAP for callus induction followed by caulogenesis and rhizogenesis.

All rooted plants were carefully washed with distilled water to remove the agar content. Further it was safely transferred to sterilized potting mixture containing sand + cow dung + red soil (1:1:2, v/v/v) sprayed with quarter strength of liquid MS media (weekly once) and acclimatized under greenhouse conditions.

3. Results and Discussion

3.1. Response of shoot tip explant

Shoot tip explants cultured on MS medium supplemented with BAP+IAA (1mg/l each) combinations induced prominent shoot proliferation with the maximum proliferation rate of 90%. The highest number of shoots (5.16 ± 0.75 shoots/explant) with the maximum shoot length of 7.06±0.19 cm was achieved (Figure 1 and 4). This result is similar to the previous finding by (Waghmare and Pandhure, 2015) [29] in *Citrus reticulata*. When the concentration of the combination was altered, concentration dependent changes in bud proliferation were noticed. On the other hand when the combination of BAP with IAA when changed to 2,4-D, the proliferation rate and percentage gets decreased indicating the importance of IAA (auxin) in shoot proliferation (Table 1).

Table 1: Effects of different concentrations of BAP, IAA & 2,4-D and their combinations on shoot production from the shoot tip explants of *Jatropha maheshwarii* after five weeks of inoculation.

PGR's (mg/L)			Shooting response (%)	Mean shoot number/Explant (X ±SE)	Mean shoot length (cm)
BAP	IAA	2,4-D			
0.1	0.5		43	1.66 ± 0.51	4.76 ± 0.30
0.5	0.2		75	3.33 ± 0.81	5.53 ± 0.44
1.0	1.0		90	5.16 ± 0.75	7.06 ± 0.19
1.5	1.0		68	2.83 ± 0.40	2.93 ± 0.23
0.5		1.0	49	1.50 ± 0.54	2.23 ± 0.20
1.0		0.5	52	3.83 ± 0.75	4.93 ± 0.27
1.5		0.5	58	4.16 ± 0.75	5.26 ± 0.25
2.0		1.0	50	1.33 ± 0.51	5.41 ± 0.40

All experiments were repeated thrice. Data are represented as mean ± standard error

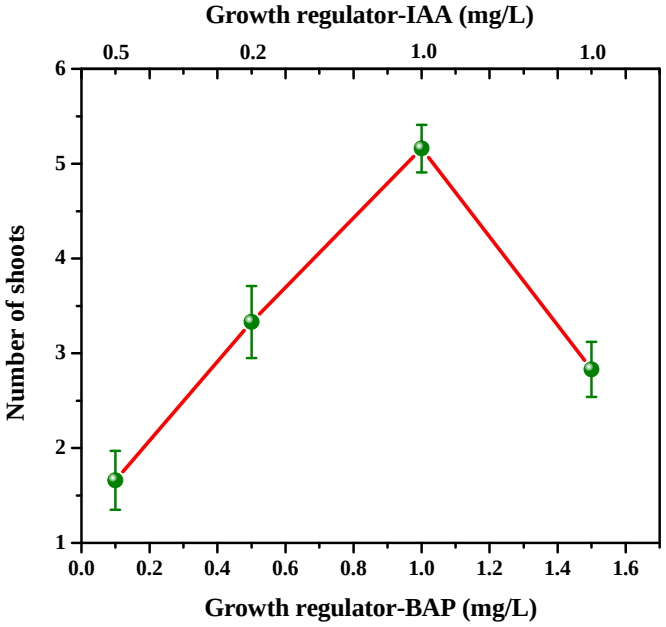

Fig 1: Effect of BAP and IAA on in vitro shoot proliferation from shoot tip explants of *J. maheshwarii*. Data were recorded after 5 weeks of culture on MS medium.

3.2. Response of axillary bud explant

While with axillary bud explants, BAP+IAA combination exhibited least response. However BAP in combination with KIN (1mg/l and 0.5mg/l) showed profused shoot budding as well as multiple shoot formation (4.33±0.51 shoots/explant) in a faster pace at 95% response rate (Figure 2 and 4). The results obtained were in corroborative with the findings of (Das and Swamy, 2015) [6] in *Atalantia monophylla*, (Sinha and Sharma, 2015) [24] in *Tinospora cordifolia* and (Singh *et al*., 2014) [23] in *Centella asiatica*. Controversially, the combination of BAP with IBA had shown prolific adventitious shoot bud induction in *Jatropha integerrima* (Sujatha and Mukta Dhingra, 1993) [25]. The data also showed that the dose response within each growth regulator combination is also distinctly different (Table 2).

Table 2: Effects of different concentrations of BAP, KIN & IAA and their combinations on shoot production from the axillary bud explants of *Jatropha maheshwarii* after five weeks of inoculation.

PGR's (mg/L)			Shooting response (%)	Mean shoot number/Explant (X ±SE)	Mean shoot length (cm)
BAP	KIN	IAA			
0.1	1.0		40	1.16 ± 0.40	2.43 ± 0.26
0.5	0.5		63	1.50 ± 0.54	5.28 ± 0.18
1.0	0.5		95	4.33 ± 0.51	4.90 ± 0.20
1.5	0.5		89	1.83 ± 0.40	3.13 ± 0.21
0.5		1.0	32	1.66 ± 0.81	1.15 ± 0.25
1.0		1.0	45	1.66 ± 0.51	1.55 ± 0.33
1.5		0.5	53	2.83 ± 0.40	4.45 ± 0.39
2.0		0.5	40	2.16 ± 0.75	3.03 ± 0.15

All experiments were repeated thrice. Data are represented as mean ± standard error

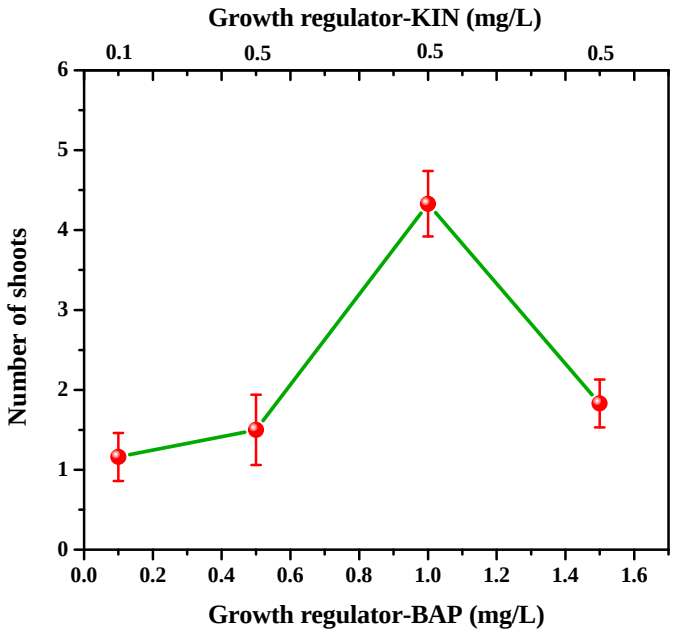

Fig 2: Effect of BAP and KIN on in vitro shoot proliferation from axillary bud explants of *J. maheshwarii*. Data were recorded after 5 weeks of culture on MS medium.

3.3. Response of leaf disc explant

Leaf disc explants cultured on MS medium supplemented with 2, 4-D (2.0 mg/l) showed callus formation all over the surface of explant within 8 days of inoculation and proliferated to a maximum growth in about 18 days (Figure 4). Similar results were also reported in previous studies by (Kaushalya and Senarath, 2013) [10] in *Gymnema sylvestre*, (Arumugam and Gopinath, 2013) [3] in *Withania somnifera*, (Pandey, 2015) [16] in *Curcuma longa* and (Jasrai *et al*., 2013) [9] in *Oroxylum indicum*. This callus further undergone caulogenesis and rhizogenesis in about 45 days. BAP when alone used also induced similar response conversely the response rate is comparatively lower (Table 3).

Table 3: Effects of different concentrations of 2,4-D and BAP on callus production, caulogenesis and rhizogenesis from leaf disc explants of *Jatropha maheshwarii* after six weeks of inoculation.

PGR's (mg/L)		Callusing (%)	Days required for callusing	Mean no. of shoots from callus (X ±SE)	% of rooted plants
2,4-D	BAP				
1.5		80	8 - 10	5.66 ± 0.51	74 ± 0.30
2.0		100	8 - 10	10.30 ± 1.03	85 ± 0.50
2.5		90	8 - 10	10.50 ± 1.22	90 ± 0.70
	1.0	70	10 - 14	7.83 ± 0.98	57 ± 0.30
	1.5	75	10 - 14	7.33 ± 0.81	55 ± 0.50
	2.0	70	10 - 14	6.16 ± 0.75	60 ± 0.50

All experiments were repeated thrice. Data are represented as mean ± standard error

3.4. *In vitro* rhizogenesis and acclimatization

Successful rhizogenesis was not achieved with the shooting medium investigated except in the case of callus derived plantlets. For this reason, the proliferated micro shoots from shoot tip and nodal explants were supposed to be subjected to inoculation on rooting medium for prominent root production.

Thus, for inducing rhizogenesis, the regenerated micro shoots from the shoot tip and nodal explants were transferred to MS medium supplemented with NAA and IBA in varied concentrations. Maximum root number (9.83±1.32) was obtained by the addition of IBA (1.0mg/l) with an average of 8.10±0.57cm root length compared with 5.33±0.81 roots in 0.5mg/l of IBA (3.60±0.47 cm) as well as 4.16±0.75 roots in 2.0mg/l IBA (4.90±0.48cm), (Figure 3). While with NAA, a maximum of 7.16 roots in 2.0mg/l hormonal concentration yielded an average of 3.30±0.40cm root length. Altogether the results revealed that NAA and IBA in low concentrations induced rhizogenesis in *J. maheshwarii*, IBA seems to be an effective hormone which responded within 10 days of inoculation with 90% rooting response (Table 4). These results were authenticated by the findings in *Jatropha curcas*, *Wrightia tomentosa* and *Gymnema sylvestre* (Shrivastava and Banerjee, 2008 [22]; Penchala *et al*., 2015 [19]; Kaushalya and Senarath, 2013 [10]). In contrast, a report on *Jatropha curcas* (Maharana *et al*., 2012) [11] had shown profuse rooting with IBA in combination with NAA. About 80 percent of the *in-vitro* regenerated plantlets were successfully transferred to soil in a green house (Figure 4).

Table 4: Effects of different concentrations of IBA and NAA on root production of in vitro grown micro-shoots of *Jatropha maheshwarii* after four weeks of inoculation.

PGR's (mg/L)		Rooting response (%)	Mean root number/Explant (X ±SE)	Mean root length (cm)
IBA	NAA			
0.5		70	5.33 ± 0.81	3.60 ± 0.47
1.0		90	9.83 ± 1.32	8.10 ± 0.57
2.0		55	4.16 ± 0.75	4.90 ± 0.48
	0.5	63	4.33 ± 1.03	2.50 ± 0.46
	1.0	35	4.50 ± 1.04	5.36 ± 0.37
	2.0	27	7.16 ± 1.60	3.30 ± 0.40

All experiments were repeated thrice. Data are represented as mean ± standard error

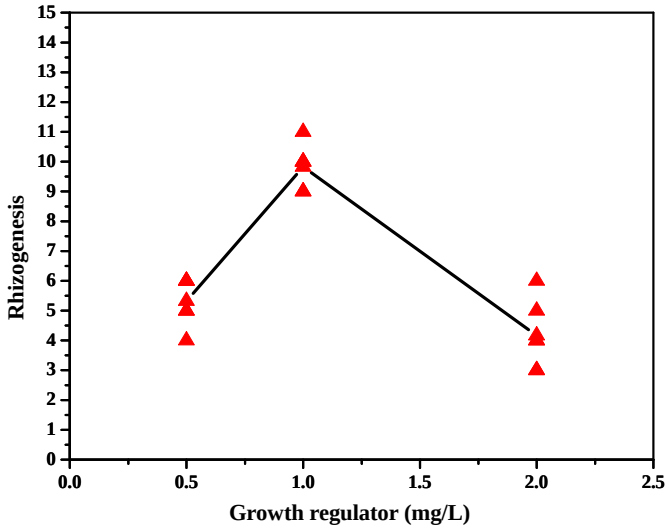

Fig 3: Dose response pattern of IBA on in vitro root induction (root number) from microshoots derived on shoot tip and nodal explants. Data were recorded after 5 weeks of culture on MS medium.

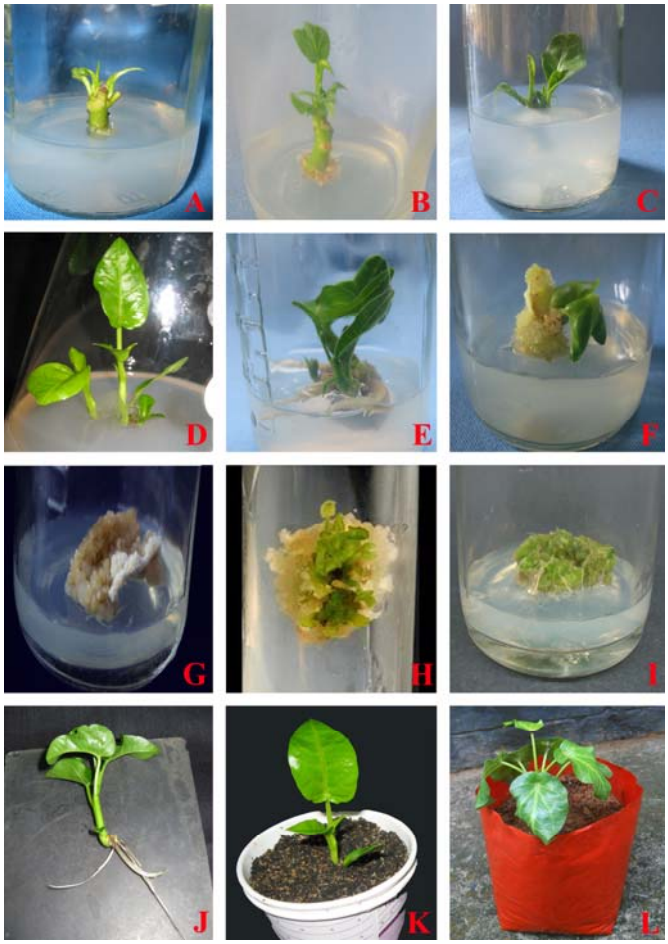

Fig 4: Micropropagation of *J. maheshwarii* (**A**-**L**). **A**-**B**: Shoot proliferation from axillary bud explants. **C**-**E**: Multiple shoot production and root formation from shoot tip explants. **F** and **G**: Callus initiation from leaf disc explants. **H**: Caulogenesis on leaf disc derived callus. **I**: Rhizogenesis noticed on leaf disc derived callus. **J** and **K**: Hardening of in vitro derived plantlet. **L**: An acclimatized plant.

Conclusion

This interesting little known endemic species having plenty of traditional medicinal potentials in it is now under severe threats due to over-exploitation, urbanization, habitat destruction and climatic change. Hence, conservation of this species is an impending need for the future. Considering this, the regeneration protocol derived here is much efficient and has the potential to propagate a good number of plantlets. Altogether, the study reveals that through direct morphogenesis, the shoot tip forms a potential explant which requires a hormonal combination of BAP and IAA for effective shoot production. While indirect morphogenesis with leaf disc explants cultured on 2,4-D was also proved to be an effective one. Thus the present investigation has resulted in the development of an eminent protocol for the mass multiplication of *J. maheshwarii* within a short time duration, which could be employed for sustainable utilization as well as conservation of the genetic resources of this valuable endemic species.

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