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INDIRECT ORGANOGENESIS AND REGENERATION OF *MACROTYLOMA UNIFLORUM* (LAM) VERDC

KARTHIKA PALANISAMY, VASANTH KRISHNAN AND DHANDAPANI GURUSAMY[1]

Molecular Biology Lab, Department of Botany, Bharathiar University, Coimbatore 641 046, Tamil Nadu, India. [1]Department of Botany, Kongunadu Arts and Science College (Autonomous), Bharathiar University, Coimbatore 641 029, Tamil Nadu, India.
E-mail: vasanthlabbu@gmail.com
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The present research was performed to develop an indirect organogenesis protocol in *Macrotyloma uniflorum* via callus by using cotyledon and epicotyledon explants. Cotyledon explants were produce the best result on MS medium supplemented with 3.0 mg/L BAP. The maximum percentage (97%) of regeneration was obtained with the combination of BAP+GA_3 (2.0+1.0 mg/L) from cotyledon explants. Well established root system was developed from regenerated shoot lets in the medium combination with BAP+NAA (1.0+1.0 mg/L). Cotyledon explants were showed more responsible than epicotyledon in terms of callus induction and plant regeneration. This *in vitro,* developed plant lets were successfully acclimatized to the pots and established in the green house. This standardized *in vitro* regeneration system will facilitate further to development of a reliable procedure to accomplish genetic manipulation in this genus.

Keywords: Cotyledon, Callus induction, Epicotyledon, Indirect organogenesis, Regeneration

Macrotyloma uniflorum (Lam.) Verdc. is a hardy pulse crop of the Fabaceae family cultivated mostly in the southern region especially Tamil Nadu, Andhra Pradesh, and Karnataka, and also in some parts of Maharashtra and Madhya Pradesh. It has been an important fodder crop since the beginning of agriculture in many parts of South Asia (Murphy and Fuller 2017). It is the most widely recovered pulse crop from the prehistoric or early historic sites in India and is also an underutilized and unexplored food legume (Reddy *et al*. 2008). Due to the many desirable characters such as tolerance to drought, salinity, and heavy metals, horse gram is considered as a potential crop for enhancing tolerance in cultivated crops (Reddy *et al*. 2008). Moreover, this species exhibits many medicinal properties such as antioxidant and antimicrobial activities and is also known to be effective in the dissolution and dislocation of kidney stones (Kawsar *et al*. 2008).

Macrotyloma uniflorum (Lam.) Verdc. have wide adaptability and thrives well under adverse climatic conditions and in poor soil (Bolbhat and Dhumal 2010). In the alarming environmental distress due to global warming and water scarcity, this crop can be considered as a future crop due to its high nutritional value and better adaptability to adverse environmental conditions. High-yielding and disease-resistant plant varieties can be developed through plant tissue culture (Kawsar *et al*. 2008). Hence this research is aimed to establish a suitable regeneration protocol and culture condition, which can be used in horse gram to make superior varieties adverse to the environmental conditions.

Tissue culture allows the production and propagation of genetically homogeneous, disease-free plant material (Chatenet *et al*. 2001). Plant tissue culture represents the most promising areas of application at present time and giving an outlook into the future. It is also essential to have sufficient mother culture and reduce the number of subcultures to avoid variation and plan the production of plants according to the demand. The *in vitro* culture has a unique has a role in sustainable and competitive agriculture and has been successfully applied in plant breeding for the rapid introduction of improved plants and it has become an integral part of plant breeding (Hussain *et al*. 2012).

In horse gram, shoot formation may occur as a result directly of explants without

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callus formation (Tejavathi and Nijalingappa, 1989) or from somatic embryos formed at the ends of leaf and immature cotyledon derived callus (Mohamed *et al*. 2004, 2005). In previous indirect organogenesis studies, multiple shoots regenerated from the callus which developed by shoot tip and cotyledonary node explants (Tejavathi *et al*. 2010). In this present study, plant regeneration was successfully established by indirect organogenesis with more number of shoots from callus which derived from cotyledon and epicotyledon explants.

MATERIALS AND METHODS

Surface Sterilization: Seeds (variety. Paiyur-2) were procured from Tamil Nadu Agriculture University (TNAU) regional research station, Dharmapuri, Tamil Nadu, India. The seeds were initially rinsed with running tap water for 3 min and rinsed with 3% sodium hypochlorite for 5 min. Later, the seeds were rinsed with distilled water thrice for 5 min, followed by rinsing with 70% ethanol for 2 min. The excess ethanol traces were removed by rinsing in distilled water thrice for 5 min. The seeds were then treated with 0.1% mercuric chloride (w/v) for 3 min and thoroughly washed the seeds three to five times with sterile double distilled water. The surface-sterilized seeds were inoculated in humidified tissue paper with sterilized petriplates and incubated at 16/8 hrs light condition for germination (25 ± 2°C with 50 μmol m−2 s−1).

Selection of explant and callus initiation: Cotyledon and epicotyls were separated from three days old germinated seedlings. The cotyledon explants (0.8 - 1.0 cm) were excised by removing the cotyledons. Epicotyls were cut into pieces of approximately 1cm. MS (Murashige and Skoog, 1962) basal medium was used for callus induction. The nutrient medium consists of inorganic nutrients, carbon sources, vitamins, iron, and amino acids essential for the development of callus.

The explants such as cotyledon and epicotyls were inoculated on MS medium containing different concentrations of plant growth regulators, such as 1.0-5.0 mg/L 6-Benzylaminopurine (BAP), 0.25-1.25mg/L Thidiazurone (TDZ), and the combination of 1.0-5.0 mg/L (BAP) and 0.5 mg/L TDZ for testing the best combination for callus induction. Thirty explants in triplicates were used in each experiment for callogenesis. The number of explants that formed callus and its response was recorded at regular intervals. Callus was sub-cultured regularly every 2 weeks.

Regeneration from callus: After 20 days, the embryonic callus was subcultured on shoot induction medium (SIM) in the culture tubes containing MS salts and vitamins, sucrose along with various concentrations of cytokinins such as (BAP) 0.5 – 4.0mg/L with 6-(γ, γ-Dimethylallylamino) purine (2iP) 0.25 - 3.3mg/L, (BAP), 0.5 - 4.0 mg/L with Picloram (PIC) 0.25 –3.0mg/L and (BAP) 0.5 – 4.0 with Gibberellic acid (GA_3) 2.5 – 3.0mg/L were used. Individual elongated shoots from cotyledonary and epicotyl explants were cultured a root induction medium comprising MS salts, vitamins, and sucrose along with various concentrations of Naphthalene acetic acid (NAA) 0.5-4.0mg/L, (BAP) 0.1 with (NAA) 0.5 – 2.5 mg/L and (BAP) 1.0 with Dichlorophenoxy acetic acid (2,4-D) 0.5 to 2.5 mg/L to optimize the ideal concentration for root induction.

In vitro grown healthy regenerated plants were washed thoroughly to remove adhered media and then transferred to small plastic cups containing a mixture of autoclaved sand, soil, and vermiculite (1:1:1). The potted plants were covered with transparent polythene bags with a minute puncture and were grown in a growth chamber at 25 ± 2ºC with 85% relative humidity for 2-3 weeks. The plants were irrigated once in 2 days. Upon new leaf growth, the plantlets were transferred to polythene bags and maintained in the greenhouse.

The analysis of variance (ANOVA) appropriate for the design was carried out to

detect the significance of differences among the treatment means. The data were statistically analyzed by the DMRT (Duncan's Multiple Range Test) (Harter, 1960) at $p<0.05$ significant level.

Histological studies: The callus was fixed in formalin, acetic acid and ethanol (FAA) in the ratio 1:1:9. After 24 hrs of fixing, the specimens were dehydrated in tertiary butyl alcohol as given by Sass, (1940). The paraffin-embedded specimens were sectioned with the help of the Rotary Microtome. The thickness of the sections was set to 10-12 µm. Dewaxing of the sections was carried out by customary procedure (Johnsen, 1940). The sections were stained with toluidine blue as per the method of O'Brien *et al*. (1964). For studying the stomatal morphology, venation pattern, and trichome distribution, parenchymal section (the section has taken parallel to the surface of the leaf) as well as clearing of the leaf with 5% sodium hydroxide or epidermal peeling by partial maceration employing Jeffrey's maceration fluid (Sass, 1940) were prepared. Glycerine mounted temporary preparations were made for macerated/cleared materials. Powdered materials of different parts were cleared with NaOH and mounted in a glycerine medium after staining, followed by different cell components were studied and measured.

RESULTS AND DISCUSSION

An efficient regeneration protocol of *M. uniflorum* has been successfully established using cotyledon and epicotyls explants. Based on Varisai Mohamed *et al*. (2005) report on cotyledon explants, the present study demonstrated and produced more shoots using cotyledon and epicotyls explants. Although, various sources of explants have been reported for regeneration, so far there is no report for regeneration from epicotyls explants. Cytokinins are necessary for plant cell division, induction of adventitious bud formation, and growth of lateral buds in the cell cycle control (Gatcia Arias *et al*. 2010). (BAP) is among the most widely used cytokinins for *in vitro* callus induction in a wide range of plant species. In the present study, successful induction of potentially organogenic callus from cotyledon and epicotyls was achieved using (BAP) 3.0 mg/L with 91% callus of 0.52 mg in weight from cotyledon explants (Fig 1) and 79.33% callus of 0.44 mg in weights from epicotyl explants (Fig 2).

The calluses are of varied morphologies, cotyledon-derived callus was compact and greenish-white whereas the epicotyl-derived callus is greenish-white with friable nature (Table 1). The fresh friable callus weighs 0.32 mg was observed in 0.12 µM (2,4-

Table1: Effect of Cytokinins on callus induction of cotyledon and epicotyledon explants of *M. uniflorum* paiyur-2 variety seeds

Explants		Cotyledon				Epicotyledon			
Plant Growth Regulators (mg/L)		Initiation of Callus (Days)	Percentage of Callus Response (%)	Callus Fresh Weight (mg)	Callus Type	Initiation of Callus (Days)	Percentage of Callus Response (%)	Callus Fresh Weight (mg)	Callus Type
BAP	1.0	14	64.4±1.29^{f}	70.00±3.05^{cd}	GW-C	15	62.3±1.85^{c}	58.3±1.45^{c}	W-F
	2.0	11	75.2±0.92^{e}	73.66±0.33^{b}	GW-C	15	73.67±2.02^{b}	67±1^{b}	GW-F
	3.0	**9**	**91.0 ±0.71^{a}**	**82.66±4.05^{a}**	**GW-C**	**11**	**79.33±1.76^{a}**	**74.67±1.45^{a}**	**GW-F**
	4.0	9	80.6±0.51^{b}	78.33±1.45^{b}	G-C	13	70.33±1.45^{b}	65.67±0.33^{a}	GW-F
	5.0	12	76±1.05^{c}	69.33±2.84^{cde}	G-C	16	63.67±0.67^{c}	61.34±0.89^{c}	GW-F
TDZ	0.25	14	72.6±1.44^{c}	61.33±0.88^{def}	YW-C	14	70±1.25^{b}	62±0.58^{b}	YW-F
	0.50	10	78.8±0.38^{b}	66.33±3.52^{bc}	YW-C	12	74.67±0.89^{a}	68.67±0.89^{bc}	YW-F
	0.75	13	68.2±1.02^{ef}	58.00±1.15^{def}	Y-F	15	65.35±1.45^{c}	57.67±2.60^{a}	YW-F
	1.0	13	65.8±0.73^{fg}	56.66±1.20^{efg}	Y-F	16	59.67±0.89^{d}	54.33±1.76^{ab}	W-F
	1.25	15	56.2±0.58^{h}	54.66±1.76^{fg}	Y-F	16	53.67±1.45^{e}	59.67±0.89^{c}	W-F
BAP + TDZ	1.0±0.5	12	46±0.71^{j}	47.33±1.45^{h}	YG-F	12	57.66±1.45^{d}	51.33±0.89^{c}	GW-F
	2.0±0.5	14	53.2±0.80^{i}	49.33±2.40^{gh}	G-F	12	62.67±1.20^{bc}	56.33±0.89^{bc}	GW-F
	3.0±0.5	10	72.8±0.86^{d}	63.33±0.88^{cde}	YG-F	10	68.67±0.89^{a}	60±1.00^{a}	GW-F
	4.0±0.5	13	69.6±0.51^{e}	60.00±0.57^{cdef}	G-F	14	65.33±0.67^{b}	57.33±1.45^{bc}	GW-F
	5.0±0.5	12	63.8±1.36^{g}	51.00±0.57^{gh}	G-F	15	61.33±.089^{c}	53.33±0.89^{a}	G-F

GW-C-Greenish white compact, **G-C-** Greenish compact, **YW-C-**Yellowish white compact, **Y-F-**Yellowish friable, **YG-**Yellowish green friable, **G-F-**Greenish friable, **WF**-Whitish friable, **GW-F**-Greenish white friable, **YW-F-**Yellowish white friable. For each treatment, 50 explants were used and repeated three times. Values represent the means ± standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.

Table 2: Effect of different concentrations and combinations of BAP on shoot regeneration from cotyledon and epicotyls

Explants		Cotyledon				Epicotyledon			
Plant Growth Regulators (mg/L)		Initiation Days	Percentage of Responses (%)	Mean Number of Shoots	Shoot Length (cm)	Initiation Days	Percentage of Responses (%)	Mean Number of Shoots	Shoot Length (cm)
BAP+2iP	0.5 + 0.25	13	54.00±3.77^{efg}	7.66±0.33^{fgh}	2.07±0.22^{ef}	12	58.67±0.67^{c}	9.67±0.89^{bc}	1.27±0.15^{d}
	1.0 + 0.5	13	61.25±4.40^{cdef}	12.67±1.00^{ab}	2.93±0.52^{cde}	09	61±0.57^{b}	3.67±0.67^{b}	1.93±0.09^{c}
	2.0 + 1.0	13	67.50±3.65^{bc}	17.33±1.45^{efgh}	4.10±0.21^{b}	**07**	63.67±1.33^{a}	15.33±0.88^{a}	3.3±0.17^{a}
	3.0 + 2.0	17	55.00±4.22^{cdefg}	8.00±1.15^{b}	3.83±0.20^{bc}	11	57±1.15^{c}	10.67±0.89^{c}	2.63±0.13^{b}
	4.0 + 3.0	17	47.50±5.26^{fg}	2.66±0.33^{fgh}	3.20±0.31^{bcd}	11	54.67±0.88^{d}	7.33±0.89^{bc}	1.6±0.17^{cd}
BAP+ Picloram	0.5 + 0.25	11	66.75±4.97^{cdefg}	15.33±1.45^{efgh}	2.10±0.21^{ef}	16	63.33±0.89b	11±100^{b}	1.2±0.11^{c}
	1.0 + 0.5	9	72.50±5.59^{cde}	18.67±0.88^{cd}	2.77±0.26^{de}	12	68±0.57a	14.67±0.89^{a}	1.93±0.08^{b}
	2.0 + 1.0	12	61.25±3.98^{defg}	11.67±1.45^{c}	3.43±0.23^{bcd}	12	62.69±0.89c	10±1.00^{c}	2.8±0.15^{a}
	3.0 + 2.0	11	53.00±4.62^{efg}	4.00±1.00^{ab}	2.77±0.15^{de}	15	58.67±0.33bc	7.66±0.89^{bc}	1.07±0.07^{c}
	4.0 + 3.0	8	49.00±5.00^{g}	3.33±0.33^{hi}	1.83±0.18^{f}	17	55.33±0.89^{d}	5.67±0.89^{c}	1.86±0.08^{b}
BAP + GA_3	0.5 + 0.25	8	67.50±3.65^{bc}	17.00±1.15^{ab}	3.96±0.88^{b}	12	74.67±1.45^{c}	17±0.57^{b}	3.1±0.20^{b}
	1.0 + 0.5	8	**83.00±4.19^{a}**	**26.00±0.57^{a}**	**5.13±0.27^{a}**	14	**77±1.15^{a}**	**19.33±0.89^{a}**	**4.23±0.14^{a}**
	2.0 + 1.0	10	76.25±3.72^{ab}	21.33+1.20^{ab}	4.06±0.39^{b}	12	72.33±1.2^{b}	15.38±0.33^{b}	3.4±0.20^{b}
	3.0 + 2.0	7	65.00±4.22^{bcd}	16.33±1.45^{c}	3.26±0.39^{bcd}	12	61.33±0.66^{c}	9.33±0.89^{c}	2.5±0.23^{c}
	4.0 + 3.0	10	61.25±3.98^{cdef}	11.67±0.88^{cd}	2.8±0.23^{de}	11	58.33±0.89^{d}	5.67±0.89^{d}	1.87±0.08^{d}

For each treatment, 50 shoots initiated on callus were used and repeated three times. Values represent the means ± standard error. Mean values followed by the same letters with in a column are not significantly different according to Duncan's multiple range test at 5% level.

D) (Tejavathi *et al*. 2010) and nature is found to be closely linked to the obtained greenish-white friable calli of cotyledon explants (4.5 µM NAA and 10 µM Zeatin) in another study (Mohamed *et al*. 2004, Zambre *et al*. 1988).

There are no reports on efficient plant regeneration from epicotyls explants of *Macrotyloma uniflorum*. In *Arachis hypogea,* 90.4 % callus induction was observed with epicotyl-explants in the combination of 0.2 mg/L (NAA) with 0.5 mg/L Kinetin from (Venkatachalam *et al*. 1998).

Various horse gram explants that have been used to regenerate plants via organogenesis such as shoot tip (Tejavathi and Nijalingappa 1989), shoot apices and cotyledonary node (Tejavathi *et al*. 2009), cotyledon (Varisai Mohamed 2005), leaf (Mohamed *et al*. 2004) and seeds (Amal *et al*. 2020). But the present study showed a differential response from the explants tested from paiyur -2 variety of horse gram using cotyledon and epicotyl explants.

Recently, Verma *et al*. (2011) reported differential shoot development response based on the concentration of (TDZ) used in the culture medium. Thus, both (BAP) and (TDZ) are effective cytokinin for shoot organogenesis in horse gram. In the present study, BAP showed effective shoot responses with cotyledon and epicotyls explants was tested (Table 2).

In line with our results, similar results were obtained for best shoot regeneration from

Table 3: Effect of auxins on root induction with different growth regulators and concentration from elongated *in vitro* shoots on callus derived cotyledon and epicotyl explants

Explants		Cotyledon				Epicotyledon			
Plant Growth Regulators (mg/L)		Initiation Days	Percentage of Responses (%)	Mean Number of Roots	Root Length (cm)	Initiation Days	Percentage of Responses (%)	Mean Number of Roots	Root Length (cm)
NAA	0.5	15	59.00±5.77^{bcd}	4.20±1.00^{abcd}	4.56±0.42^{bcde}	24	59.83±1.01^{b}	2.33±0.33^{b}	3.07±0.18^{c}
	1.0	18	68.33±3.33^{ab}	5.66±0.57^{abc}	5.43±0.29^{b}	21	65±0.87^{a}	5.6±0.33^{a}	4.77±0.15^{a}
	2.0	18	65.00±0.00^{bcd}	4.33±1.20^{abcd}	4.63±0.29^{bcde}	21	63.33±0.89^{b}	4±0^{b}	3.73±0.20^{b}
	3.0	16	57.66±3.33^{cd}	3.00±1.52^{bcd}	3.76±0.31^{defg}	23	52±0.58^{c}	3.33±0.33^{b}	2.8±0.15^{a}
	4.0	15	52.00±5.77^{de}	2.43±0.33^{d}	2.96±0.14^{fg}	23	20.33±1.45^{d}	1±0^{c}	1.53±0.14^{d}
BAP+NAA	1.0+0.5	15	61.00±0.00^{bcd}	4.00±1.15^{abc}	4.10±0.66^{cdef}	21	66.67±0.88^{b}	4.67±0.89^{b}	2.9±0.15^{d}
	1.0+1.0	17	**73.33±3.33^{a}**	**7.00±1.00^{a}**	**7.40±0.32^{a}**	22	**69.67±0.88^{a}**	**5.67±0.33^{a}**	**6.33±0.08^{a}**
	1.0+1.5	16	68.33±3.33^{bcd}	6.0±0.88^{abcd}	5.03±0.18^{bcd}	21	57.83±0.60^{c}	3.67±0.33^{a}	5.43±0.17^{b}
	1.0+2.0	16	67.00±5.77^{bc}	5.66±0.57^{ab}	4.50±0.40^{bcde}	21	42.33±0.89^{d}	2.66±0.33^{b}	4.43±0.18^{c}
	1.0+2.5	13	57.66 ± 6.66^{cd}	3.00±1.73^{bcd}	3.56±0.38^{efg}	18	31.67±0.89^{e}	1.85±0.58^{c}	3.13±0.09^{d}
BAP + 2,4-D	1.0+0.5	22	52.00 ± 5.77^{f}	2.33±0.33^{cd}	2.60±0.30^{g}	19	52.5±0.76^{c}	1.33±0.33^{d}	1.67±0.19^{d}
	1.0+1.0	22	59.33 ± 5.77^{ef}	3.33±1.20^{abcd}	3.90±0.37^{cdef}	19	57±0.58^{d}	2±0.00^{c}	3.23±0.78^{c}
	1.0+1.5	24	71.00 ± 0.00^{bcd}	5.00±0.57^{abc}	4.13±0.63^{cdef}	22	68.83±0.44^{c}	4.53±0.33^{b}	3.9±00.06^{a}
	1.0+2.0	21	67.55±0.00^{bc}	4.66±0.88^{abcd}	5.13±0.35^{bc}	24	65.5±0.76^{a}	3±0.00^{a}	3.2±0.11^{b}
	1.0+2.5	21	64.66 ± 3.53^{cd}	3.66±0.66^{abcd}	3.40±0.30^{efg}	20	60.5±1.26^{b}	2.60±0.33^{b}	2.33±0.09^{c}

For each treatment, 50 elongated shoots were used and repeated three times. Values represent the means ± standard error. Mean values followed by the same letters within a column are not significantly different according to Duncan's multiple range test at 5% level.

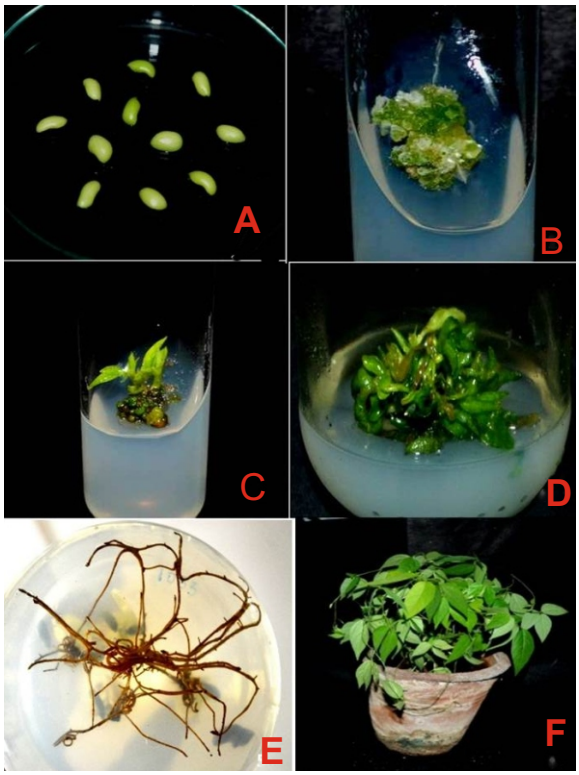

Figure 1 (A-F): - Indirect organogenesis of Horse gram, *Macrotyloma uniflorum* Var. Paiyur- B**.** using cotyledon explants **A.** Cotyledon explants prepared from *in vitro* raised seedlings 2) Nodular and green compact callus induction on MS medium BAP (3.0 mg/L) **C.** Adventitious shoot bud formation on organogenic callus **D.** Proliferation of multiple shoots in BAP+GA3 (1.0+0.5 mg/L) **E.** Regenerated shoots produce roots in BAP + NAA (0.2+1.0 mg/L) **F.** Hardened plants

the callus of *P. vulgaris* on medium supplemented with 5.0 mg/L of (BAP). Also, other reports affirm that (BAP) is effective for shoot induction in *P. vulgaris* (Arellano *et al*. 2009, Mukeshimana *et al*. 2013).

When 0.5 mg (TDZ) were used alone in the MS medium, 0.66 mg of callus fresh weight was observed within 10 days and when (TDZ) is used with a combination of (BAP), the shoot formation is observed from callus. Collado *et al*., 2013 report that the highest callus weight was observed within 21 days in the concentration of 0.04 mg/L (TDZ) with Gamborg B5 medium. But unfortunately, the callus was turned dark brown when the same concentration of hormone was used for shoots induction from callus.

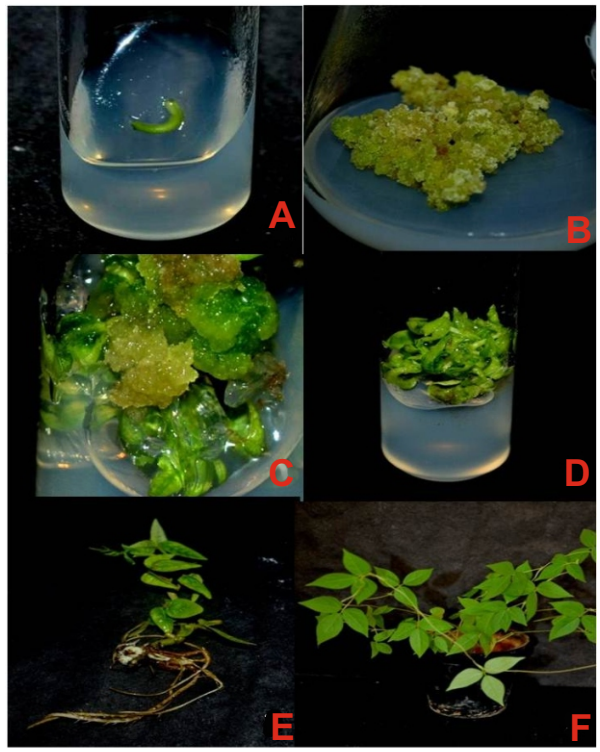

Figure 2 (A-F):- Indirect organogenesis of Horse gram, *Macrotyloma uniflorum* Var. Paiyur-2 using epicotyl explants **A.** Epicotyl explants from *in vitro* germinated seeds **B.** Callus production from Epicotyl explants **C.** Differentiation of induced callus tissue into multiple shoot buds **D.** Proliferation of multiple shoot buds **E.** Elongated shoot buds produced root system **F.** *In vitro* grown seedlings established in the pot

Shoot Regeneration and Elongation: Callus cultured on multiple shoots induction medium containing (BAP+GA_3) 1.0+0.5 mg/L) started to produce adventitious shoot bud formation along with small nodules on organogenic callus pieces of cotyledon explants within 28 days and epicotyledon explants within 31 days. The light green areas of the calli were most competent for adventitious shoot bud differentiation. A cluster of shoot primordia formed in the nodule area and leaf-like structures emerged from the shoot primordia. Maximum shoots of 26 with an average length of 5.13cm and regeneration response of 83% was observed from callus of cotyledon explants and 19 shoots with an average length of 4.23cm and regeneration response of 77% were observed with epicotyls explants supplemented with (BAP+GA_3)

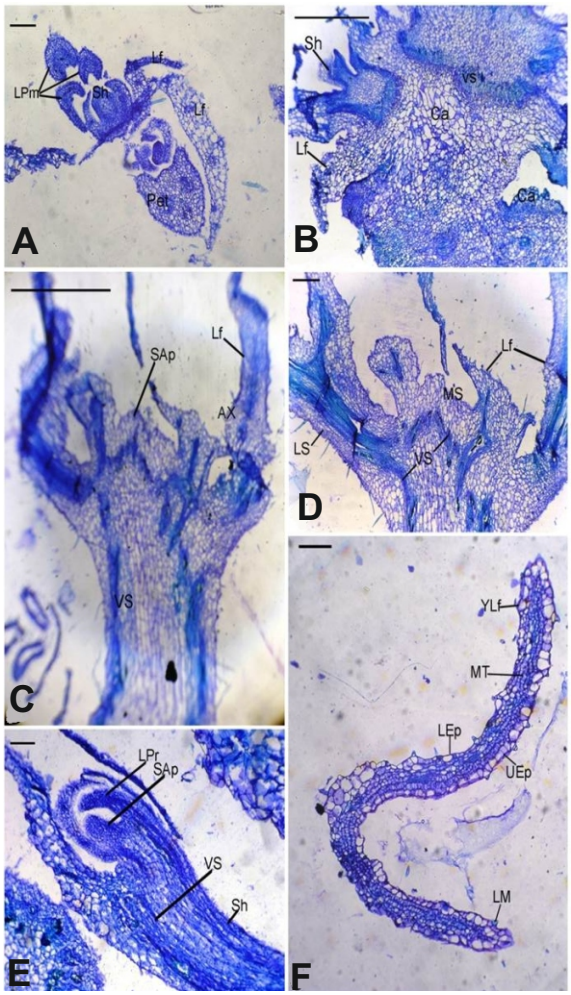

Figure 3(A-F):-- Histological section of Horse gram, *Macrotyloma uniflorum* Var. Paiyur-2 regenerating callus showing initial development of shoot buds **A.** T.S of shoot apex of young shoots developed by the callus. **B.** T.S of callus showing vascular cylinder and axillary formed in the axil of a leaf. **C.** Callus masses from which a shoot complex develops. **D.** L.S of shoot complex formed from the callus. **E.** L.S of young shoot, with shoot apex and leaf primordia. **F.** T.S of young leaves produced by the callus. Abbreviations: LPm-Leaf Primordia; Pet-Petiole; Sh-Shoot Primordia; VS-Vascular Strand; Ca-Callus; Lf-Leaf; Ax-Axis of the Shoot; MS-Main Shoot; SAp-Shoot Apex; MT-Mesophyll Tissue; UEp-Upper Epidermis; LEp-Lower Epidermis; LM-Leaf Margin; Ylf- Young Leaf

1.0+0.5 mg/L (Table 2). However, as the concentrations of both BAP and GA3 increased, the regeneration efficiency was decreased gradually. The present study reported more efficient and producible outcomes when compared to similar studies reported earlier (Tejavathi *et al*. 2010; Venkatachalam *et al*.1988).

It is suggested that (GA_3) along with the cytokinin stimulates the development of shoot primordial induced by (BAP) (Ghulam *et al*. 2017). Adding GA_3 to (BAP) containing media at any concentration has been reported to improve the frequency of somatic embryogenesis (Li *et al*. 2002). GA_3 stimulates the production of numerous enzymes, notably α-amylase, in germinating cereal grains. Besides, (GA_3) Gibberellic acids promote seed germination in some species that otherwise require cold stratification and/or light for inducing seed germination (Davies, 1995). Experimental results suggest that the *Gibberellin Responsive* gene (*HvGR*) (Localized to pockets of subepidermal cells from where shoot primordial originate) is responsible for (GA_3) induced shoot induction. (*HvGR)* shows a high level of expression in the regeneration stage of the initiated shoots where the signal was localized primarily in the developing shoot primordial (Seong *et al*. 2004).

Shoot regeneration was also induced from cotyledon and nodal explants for six cultivars from other regions of the world (Hamdy and Hattori, 2006a, b). The percentage of adventitious shoot development was varied from 0-55.1% depending on the cultivar, type and concentration of growth regulators (Hamdy and Hattori 2006a). Edyta *et al*. (2012) reported along with (NAA) and (BAP) supplementation of (GA_3) was increases the callus viability and shoot regeneration by independent of genotype and tannin content, in contrast to other reports (Hamdy and Hattori, 2006, Bahget *et al*. 2008).

Rooting and Acclimatization: The recalcitrant nature of legumes to rooting in culture conditions has limited the successful application of any biotechnological approaches for crop improvement. The auxins (2,4-D) and (NAA) at various concentrations are commonly used to induce rooting from the

explants in both *in vitro* and *in vivo* cultures. Rooting is much favored when auxins are used along with cytokinins whereas 73.33% (Table 3) root formation (7 roots of 7.40cm length) was observed in the callus derived from cotyledon explants and in the case of epicotyl derived callus, 69.67% root formation was observed with 5.67 roots of 6.33cm in length when treated with 1.0mg/L of (BAP) and 1.0mg/L (NAA) within 17 and 22 days after exposure to rooting medium, respectively.

Regeneration of shoots from both cotyledon and epicotyls was found to occur via the indirect organogenesis method. It is noteworthy that more shoots were differentiated on the cotyledon-derived callus compare to the epicotyl-derived callus. It indicates that lower cytokinin concentrations and a significantly higher amount of auxin ratio were suitable for root induction. The well-rooted plantlets were transferred to plastic cups containing sterile sand, soil, and vermiculite (1:1:1 v/v/v). 84% percent of the plantlets that survived during acclimatization and acclimatized plants (Fig 1&2(6) were transferred to the greenhouse. The regenerated plants did not show detectable variation in morphological or growth characteristics compared to the parent plant. Whereas reports show that about 60% of survival plants were achieved using soil:sand:manure (1:1:1) (Tejavathi *et al*. 2010) and about 55% of survival plants were achieved using Vermiculite: sand and red soil mixture (1:1:1) was tested (Mohamed *et al*. 2005).

In vitro induction of organogenesis depends on the endogenous concentration of plant growth regulators, their distribution in the cultured tissue, and interaction with an exogenously supplied growth regulator. Efficient (83%) shoot organogenesis was observed using (BAP) in combination with (GA_3) from cotyledon derived callus as the similar effect of this combination was noted previously (Ananthakrishnan *et al*. 2003).

To date, there are reports available on the culture of horse gram with various explants derived from the reliable protocol for mass multiplication of plantlets (Tejavathi *et al.*, 2010). It has been suggested that the tissue culture of legumes from cotyledon explants has been regarded as a good explants source. In this study, were successfully produced from the embryogenic callus of *M. uniflorum* from cotyledon and epicotyl explants in the indirect organogenesis method.

The explants (young petals and leaves) carrying different sets of epigenetic regulators will probably decide the regeneration conditions (for example, exogenous amino acids and plant growth regulators in media) and affect the regeneration outcomes (for example, cytosine methylation variation in regenerates) by mediating responses to external stimuli and initiating epigenetic alterations that influence adaptation to stresses (Miguel and Marum 2011).

With proper selection of explants and optimization of the culture medium and hormones, *in vitro* micropropagation of this plant can be carried out successfully. The media described in this paper for horse gram regeneration was independent of the genotype.

Histological studies: Histological observations revealed that the origin of shoot buds directly from the cultures of cotyledon and epicotyls raised on (MS+BAP) 3.0 µM. As the callus grows, it produces small shoots at several points of the callus. The shoot primordial has shoot apex with young leaf primordial. The leaf primordia are curved with shortleaf lamina. The mature leaf produced a well-developed lamina with homogenous mesophyll tissue and lateral veins. The callus has a well-differentiated vascular cylinder with xylem and phloem. When the shoot develops from the callus, the vascular traces originate from the main vascular cylinder of the callus and enter into the shoot axis. From the shoot axis, the vascular traces enter into the leaves to form the veins. The young leaves have a small collateral vascular bundle and wide

parenchymatous shows ground parenchyma cells. Small non-glandular trichomes are common in the outer (lower) surface of the lamina. The shoot apical meristem has cytoplasmic callus forming lateral initials of leaf primordial and it was grow into mature leaves which possess district midrib and lateral veins, young leaves are folded into V- or U-shaped outline (Fig 3).

CONCLUSION

This study developed an efficient and reproducible regeneration protocol via indirect organogenesis from the cotyledon and epicotyl explants of horse gram, var. paiyur-2. Freshly collected seeds are optimal for callus formation without affecting the regeneration capability of calli with anatomical proof. The formation of calli strongly determines the explant's type and seed age. The number of shoots per callus was dependent on both the concentration of (BAP) used during callus proliferation and the combination of (BAP) with (GA_3) concentration used for shoot formation and elongation. The regeneration frequency that we reported is high when compared with the results obtained in the previous studies. The present study concluded that established protocol applies to two different explants and may therefore be used to regenerate other genotypes. The results presented here are expected to be helpful for genetic transformation study to improve the traits of the cultivars.

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