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Optimization of *in vitro* micropropagation of ethnomedicinally important underexplored plant *Maytenus emarginata*

MAHENDRA SINGH[1], VIMALA YERRAMILLI[1], ISHWAR SINGH[1]* and DEEPTI SINGH[2]

Chaudhary Charan Singh University, Meerut 250 004, India

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ABSTRACT

In vitro callus induction and shoot emergence protocols for *Maytenus emarginata* (Willd) Ding Hou. have been established using young leaves and stem explants of *in vivo* growing, one and half year-old plants. Plants were growing in the month of August 2016 at department of Botany, Ch. Charan Singh University, Meerut. However, explants were collected in the month of August, 2019. MS (Murashige and Skoog) medium and WPM (Woody Plant Medium), supplemented with different concentrations and combinations of plant growth regulators (PGRs) such as 0.5-2.0 mg/l Naphthalene acetic acid (NAA), 0.5-1.0 mg/l Benzyl Amino Purine (BAP), 2, 4-D (1.0-2.0 mg/l) and 1.0-1.5 mg/l Gibberellic acid (GA_3) and temperature regimes were used for callus induction and shoot emergence. The presence of GA_3 was found necessary for callus induction and shoot emergence from both explants (leaf and stem). The best response of callus formation from leaf explants was obtained at 24°C on WPM supplemented with 0.5 mg/l NAA+1.0mg/l BAP+1.5mg/l GA_3 and MS medium supplemented with 2.0 mg/l NAA+ 0.5 mg/l BAP+ 1.0mg/l GA_3. Stem explants exhibited best degree of callus induction only on MS medium supplemented with 0.5mg/l NAA+ 1.0 mg/l BAP+1.5 mg/l GA_3. Light and temperature play very crucial role in shoot emergence because exposure of callus or nodal section of stem, to 12 hours dark period followed by 12 hours PAR (photosynthetically active radiation) at 27±2 °C induced shoot emergence on MS medium supplemented with 0.5mg/l NAA+1.0mg/l BAP+1.5 mg/l GA_3 after 42 days. This is the first optimization report on tissue culture and medicinal use of callus of *Maytenus emarginata*.

Keywords: BAP, Callus, GA_3, *Maytenus emarginata*, NAA, Shoot emergence

Maytenus emarginata (Willd) Ding Hou., is a member of the family Celastraceae, globally distributed throughout in Paleotropics. In India, it is commonly found in dry scrub forests of Gujarat and Rajasthan (Sangwan *et al*. 2011). It tolerates various types of adverse conditions such as drought, salinity, heat and cold (Rathore *et al*. 1992). Quinone-methide-triterpenes (QMTs), a distinctive group of triterpenoids, are considered as chemotaxonomic indicators, of family Celastraceae (Paz *et al.* 2013), reported in all *Maytenus* species making this genus medicinally important and mainly used for treatment of inflammatory diseases (Veloso *et al*. 2017), antitrypanosomal (Dos *et al*. 2013), antifungal (Gollu *et al*. 2012), antioxidant (Dos *et al*. 2010), anti-inflammatory (Kim *et al*. 2009, Khichar *et al*. 2019). Maytenin, an important QMT of *Maytenus* sp. has been reported to show anticancer activity against various types of cancer cell lines such as lung, breast, colon, cervix, melanoma, myeloma, glioblastoma, leukemia, prostate, vinblastine-resistant, etc. (Seo *et al.* 2011, Lei Zhang *et al*. 2020), Oramas-Royo *et al*. 2010). *In vitro* shoot multiplication and root formation from nodal explants of *Maytenus emarginata* has been reported by some workers (Rathore *et al.* 1992, Mathur and Goswami 2014), where explant was young seedling or nodal stem region. However, in the present investigation, callus induction from young leaf and stem explants to avoid non-availability or non-germinability of seeds, for isolation of bioactive compounds as a non-destructive method for the green cover on earth and *in vitro* direct organogenesis for plant regeneration/ multiplication have been our focus of research. It responds to minute changes in temperature and PGRs. Hardening of this plant in different climatic conditions is also a challenge. This medicinally important bushy plant grows slowly, requires a huge amount of nutrient medium and its population is dwindling in its native places due to rapid urbanization. It may be used for afforestation of those sites where normally plants cannot be grown easily.

MATERIALS AND METHODS

Plants were collected from Gujarat in the month of June, 2016 and planted in the Department of Botany Ch. Charan Singh University, Meerut. After appropriate rooting and establishment of plants in our local condition, healthy young leaves and stem explants were collected in the

[1]CCS University, Meerut; [2]Meerut College Meerut.
*Corresponding author e-mail: ishwarsingh@gmail.com.

month of August, 2019 for in *vitro callus* induction and micropropagation respectively.

Explant preparation for inoculation: Explants were cleansed with Labolin in beaker, thoroughly washed under running tap water for 30 minutes and finally rinsed 3 times with sterile distilled water. Under laminar air flow hood, explants were cut into small pieces of not more than 1.0 cm. For surface sterilization, these cut pieces were dipped in 1.0% Bavistin for 5 min., washed twice with sterilized distilled water, then immersed in 0.1% mercuric chloride solution for 4 minutes, thorough rinsed twice with sterilized distilled water before inoculation.

Media preparation: MS (Murashige and Skoog 1962) and WPM (Woody Plant Medium) (Lloyd and Mc Cown 1980) of HI Media Company were used for induction of callus for the preparation of medium. Ready media dry powders of MS medium and WPM medium contained all essential vitamins, minerals and sucrose. Agar-agar was added separately in WPM. Various concentrations and combinations of plant growth regulators (PGRs) like 2,4-D, IAA, NAA, Kinetin, BAP and Gibberellins, were added in MS and WPM medium. Each flask with at least 40 ml medium (required for morphogenetic changes), was plugged tightly with non-absorbent cotton plug. Flasks were further wrapped and tightened with rubber band to protect from condensation of water during autoclaving followed by marking each flask with concentration and combination of PGRs. The media in these flasks were sterilized in autoclave at 121ºC and 15 lb psi for 15 minutes, followed by maintenance at room temperature in the culture room for 2-5 days before inoculation. Inoculation in culture flasks containing WPM or MS basal medium supplemented with different concentrations of Auxin (NAA/IAA/2,4-D) cytokinin (BAP/Kn) and GA_3 were carried out in 30-50 minutes pre-UV sterilized laminar air flow chamber.

Observations

Callus induction: Callus induction and growth of callus were observed in all the flasks containing young leaf and stem explants on different concentrations and combinations of growth regulators within 30 to 40 days. The best callus induction medium was selected for further multiplication of unorganized mass. Calli were sub-cultured for further multiplication after 5 to 10 weeks of callus growth, on fresh WPM and MS basal medium with a series of combinations of growth regulators as the response on individual growth regulator was very poor. However, best callus induction and growth on WPM/MS medium with a combination of PGRs showed similar type of callus induced in 35 days (in WPM) to 40 days (in MS). In WPM higher NAA to BAP ratio (4:1) resulted in 80% callus induction response, whereas in MS higher BAP to NAA ratio (2:1) resulted in better degree of callus growth in 90% flasks. Additionally, increased amount of GA_3 (1.5 mg/L) was required with MS, whereas on WPM with lower amount of GA_3 (1.0 mg/L) callus induction and growth response were noted which did not sustain an increase up to 1.5 mg/l GA_3. Leaf

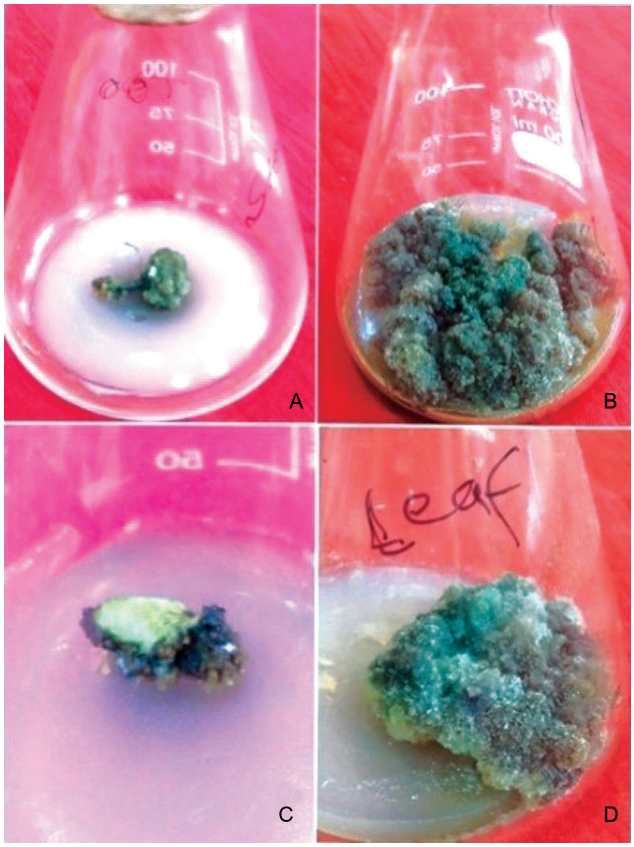

Fig 1 A, Initiation of callus from stem explant; B, subculture of callus of stem explant; C, initiation of callus from leaf explant; D, subculture callus of leaf explant; E.F, initiation of regeneration from stem explant; G.H, subculture regenerated stem explant with root initiation.

explants responded to 2,4-D substituted in place of NAA on MS media, although the callus induction and growth was comparatively quite poor. Interestingly, incubation conditions played an important role in response of callus growth or organogenesis. (Table 1, Fig 1).

The inoculated culture flasks were incubated for 48 hours dark cycle followed by12 hours light and 12 hours dark cycle at 25±2°C for callus induction. For shoot emergence (direct organogenesis from nodal explants), culture flasks with nodal explants were incubated at 27±2ºC and 12 hours light and 12 hours dark cycle.

Callus from stem explants responded like leaf explants largely. However, there was no difference in response to replacement of NAA to IAA. The calli developed were light brown as against light green from leaf explant. On MS media stem explants resulted in compact light green calli although with the same 90% ratio of induction. (Table 1)

Shoot emergence

For shoot emergence, temperature and light played a unique role. It has been noticed that in all the flasks containing different explants on different concentration and combination of growth regulators at 27±2^{0}C temperature and 48 h dark period followed by PAR exposure of 12 h

Table 1 Effect of PGRs on callus induction from leaf and stem explants of *M. emarginata*at 25±2°C and 12 hours alternating PAR and dark cycles

Explant	Nutrient medium	PGRs, mg/L	Days	Degree of response	Percent response	Callus colour	Nature of callus
Leaf	WPM	1.0 NAA + 0.5 BAP + 1 GA_3	35	+ +	70	Light green	Friable
Leaf	WPM	2.0 NAA + 0.5 BAP + 1.0 GA_3	35	+ + +	80	Light green	Friable
Leaf	MS	0.5 NAA + 1 BAP + 1.5 GA_3	40	+ + +	90	Light green	Friable
Leaf	MS	1.0 NAA + 0.5 BAP + 1.5 GA_3	40	+ +	70	Light green	Friable
Leaf	MS	1.0 2,4-D + 0.5 BAP + 1.5 GA_3	40	+	30	Light green	Friable
Stem	WPM	1.0 IAA + 0.5 BAP + 1.0GA_3	40	+ +	70	Light brown	Friable
Stem	WPM	1.0 NAA + 0.5BAP + 1.0GA_3	40	+ +	70	Light brown	Friable
Stem	MS	0.5NAA + 1.0 BAP + 1.5 GA_3	40	+ + +	90	Light green	Compact

Table 2 Effect of PGRs on direct shoot emergence from nodal explants at 27±2^{0}C and 12 h alternating PAR and dark cycles

Explant	Nutrient medium	PGRs, mg/L	Days	Degree of response	Percent response
Nodal explant	WPM	2.0 NAA + 1.0 BAP + 1.0 GA_3	45	2	70
Nodal explant	WPM	0.1NAA + 2.5BAP + 1.0GA_3	45	1	30
Nodal explant	MS	1.0 NAA + 0.5 BAP + 1.5 GA_3	42	2	70
Nodal explant	MS	0.5 NAA + 1 BAP + 1.5 GA_3	42	3	90
Nodal explant	MS	1.0 NAA + 1.0 Kn	40	1	30

alternating with 12 h dark period, led to shoot emergence after 40 to 45 days. The best PGR combination for shoot emergence was selected for further multiplication of shoots. (Table 2)

Regenerated tissue was sub-cultured on fresh WPM and MS basal medium on the same concentration and combination of growth regulators for 5 to 7 weeks under aseptic conditions in laminar air flow chamber, resulting into callus induction. Longer exposure of differentiated tissue to T1 (25±2°C) condition in dark led to blackening of the tip, similarly longer exposure of the callus to light and T2 (27±2°C) temperature led to blackening and death of the callus, if the conditions were not reverted within one week of initiation of blackening.

RESULTS AND DISCUSSION

Maytenus emarginata plant parts (young leaves and stem) have the potential to dedifferentiate into callus and re-differentiate into plantlet. However, temperature variation of barely 2ºC decides critically, differentiation to plantlet (27ºC) or dedifferentiation to callus (25ºC). Besides, light is also crucial in proper development of regenerated plantlet. *Maytenus* generally grows in nature in temperate region and Gujarat state of India, during July and August season. In our culture experiments to 12 h cycle of light (PAR) and dark periods resulted in callus induction/organogenesis response. The soil moisture and relative humidity of air are 65–72% and temperatures range between 29–35°C (Table 3). Amongst PGR combinations supplemented, NAA was more effective over 2-4, D and BAP was more effective against kinetin and further supplementation with GA_3 This suggests the meristematic activity for callus/ plantlet growth is arrested till GA_3 is supplied in differential amount to leaf (1.5 mg/L) or stem (1.0 mg/L) for breaking the dormancy. Khan *et al*. (2015) have also used GA_3 for shoot regeneration from grape callus cultures. However, in our experiment, no regeneration from callus cultures could be attained, instead, micro-nodal explants directly exhibited shoot emergence and plantlet growth. Further, meristematic activity has been found to be controlled by moderate temperatures, as in August to November in Gujarat, if maintained under moderate temperatures with prior exposure to dark period. However, negligible increase in temperature could induce plantlet differentiation (regeneration) instead of callus induction. With exposure to 16 hours light period just after an initial 2 days dark period, dark period is necessary

Table 3 Effect of temperature on plantlet regeneration and callus induction from regenerated plantlets on MS media

PGR concentration (mg/L)	Plantlet regeneration		Callus induction	
	T1 (25 ± 2°C)	T2 (27 ± 2°C)	T1 (25 ± 2°C)	T2 (27 ± 2°C)
0.5 NAA + 1 BAP + 1.5 GA_3	–	+	+	–
1 NAA + 0.5 BAP + 1.5 GA_3	–	+	+	–
1.0 2,4,D + 0.5 BAP + 1.5 GA_3	–	+	–	–
1.0 NAA + 0.5 BAP + 1 GA_3	–	+	+	–
2.0 NAA + 0.5 BAP + 1.0 GA_3	–	+	+	–

because dark period of few day before exposure to light is beneficial for plants that are prone to synthesized phenolics (Dhar and Upreti 1999), results in blackening of tips of regenerated plantlets and callus. This indicates that premature exposure to high PAR results in stress leading to bypass pathway synthesizing phenolics and tannins, which inhibit growth. If the PAR exposure is limited to 12 hours good callus growth at 25±2ºC and plantlet regeneration at 27±2ºC could take place. Similar effects of photoperiod and variable temperature have been noted by Tao *et al*. (2012) during callus differentiation in *Swertia mussotii*.

A protocol for *in vitro* callus induction and shoot emergence, for *Maytenus emarginata*, using leaf and the nodal section as explants respectively was developed. Temperature and light act as a limiting factor for callus induction and shoot emergence beside ratio of NAA to BAP and the presence of optimal GA_3. This is the first report for *in vitro* callus induction, shoot emergence from *in vivo* growing plants and the effect of GA_3, light, and temperature on callus induction and shoot emergence on WPM and MS. This work will form a basis of non-destructive method of callus formation for isolation of medicinal principles unique to this plant. There are only a few research papers available online for the *in vitro* study of this plant. A full biochemical screening of callus is also needed to elucidate the most active compound responsible for the treatment of critical diseases using leaves by tribals of Gujarat. 25±2ºC with12 hours light, 12 hours dark cycle and 27±2ºC temperatures with 12hours light and 12 hours dark cycle were favourable for callus induction and shoot emergence, respectively, indicating the two phenomena to be governed differently by phytochromes and have distinct photoperiodic response. Callus induction and shoot emergence were obtained at 5 to 7 weeks and 5 to 10 weeks, respectively, which need further understanding of the differential resilience of undifferentiated and differentiated tissue system.

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