

In vitro* Plantlet Regeneration from Callus Culture of *Trachyspermum copticum

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Abstract

The effect of explant sources and plant growth regulators on callus induction and plantlet regeneration of *Trachyspermum copticum* were explored. Different explants including hypocotyl, cotyledonary node and leaf were cultured on MS supplemented with different combinations and concentrations of plant growth regulators including 2,4-D (0.2-3 0.5 mg/l), NAA (2 mg/l), BAP (1-3 mg/l), Kn (0.5 mg/l) and IAA (0.8 mg/l). The best response for callus induction (100%) as well as quality was observed from cotyledonary node segments cultured on MS supplemented with 2, 4-D at 1 mg/l in combination with Kn at 0.5 mg/l. Calli derived from various explants were subcultured on shoot induction media with different compositions and concentrations of medium. MS without any plant growth regulator promoted the highest frequency of shoot regeneration (100%) and also mean number of developed shoots per explants (3.8) showed the same result. Regenerated shoots were then rooted on three-fourth strength MS with 75% efficiency after 30 days.

Introduction

Trachyspermum copticum (Umbelliferae/Apiaceae) is an annual herbaceous, 30 - 70 cm in height, which grows in eastern India, Iran, Pakistan, Afghanistan and Egypt, with white flowers and small brownish seeds (Khajeh et al. 2004, Sharifi-Rada et al. 2013, Rasooli et al. 2008). Some biological effects of *T. copticum* such as anti-inflammatory (Thangam et al. 2003), antifungal (Rasooli et al. 2008), antiviral

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(Hussein et al. 2000), anti-nociceptive (Hejazian et al. 2008), anti-filarial (Mathew et al. 2008), antioxidant activity (Bera et al. 2004), analgesic (Kaur et al. 2009), antimicrobial (Sharifi-Rada et al. 2013) and also several therapeutic effects including anti-tussive (Devasankaraiah 1974), anti-vomiting (Gilani et al. 2005), digestive stimulant (Boskabady et al. 2003), hepatoprotective (Srivastava et al. 1999) and lactogenic (Shaukat et al. 2004) have been documented.

The *in vitro* propagated medicinal plants furnish a ready source of uniform, sterile, and compatible plant material for mass multiplication and germplasm conservation of rare, endangered and threatened medicinal plants (Anis and Faisal 2005, Uppendra et al. 2005). The loss of biodiversity and plantations due to deforestation in combination with the demand from both domestic and export markets have led to the utilization of *in vitro* methods of propagation as compensating tools to meet the commercial needs (Shaik et al. 2011). Furthermore, genetic improvement is another approach to augment the drug yielding capacity of the plant (Karuppusamy et al. 2006) which depends on the establishment of efficient adventitious shoot regeneration systems (Shaik et al. 2011).

Up to date, there is no report on callus induction and regeneration of *T. copticum*. Only, a reliable protocol for micropropagation in *Carum copticum* from apical bud explants which was reported by Salehi et al. (2014). They studied the effects of several plant growth regulators, including BAP, IAA and IBA, in order to obtain high shoot and root regeneration rate and also considerable survival percentage in this species. The maximum shoot regeneration frequency (97.5%) and the highest number of shoots were gained from apical buds (34 shoots per explant) on MS fortified with BAP (4.4 $\mu\text{mol/l}$) and IAA (0.5 $\mu\text{mol/l}$). Application of IBA (6 $\mu\text{mol/l}$) in MS resulted in the rooting rate of 100%.

The objective of this study is to introduce an efficient and reproducible regeneration protocol from callus cultures of this medicinal plant. For that purpose, the effect of different explant types and plant growth regulators on embryogenic callus induction and plantlet regeneration were studied.

Materials and Methods

The mature seeds of *Trachyspermum copticum* kindly provided by Iranian Biological Resources Center Seed Bank (Accession No: IBRC P1006719, Field No: 503-6) were used. The seeds were washed thoroughly under running tap water for 30 min. The soaked seeds were treated with 70% ethyl alcohol for 1 min and 0.5% (v/v) sodium hypochlorite for 10 min and then rinsed three times with sterile distilled water (Salehi et al. 2014). The surface sterilized seeds were cultured on MS medium and incubated at $26 \pm 2^\circ\text{C}$ in darkness for germination.

The cultures were used as a source of plant material for establishment of explants before initiating the experiment.

Aseptically excised hypocotyl, cotyledonary node and leaf explants (Fig. 1b) (hypocotyls and leaf of 5 - 7 mm, cotyledon node of 3-5 mm) from 40-days-old seedlings (Fig. 1a) were cultured on sterile MS medium supplemented with different concentrations and combinations of plant growth regulators such as 2,4-D, NAA, IAA, BAP and Kn for embryogenic callus induction.

The embryogenic calli induced from various explants were subcultured two times on the same medium in every 4 weeks and subcultured. In order to evaluate regeneration, the calli were transferred to MS, half strength MS, and three-fourth strength MS without any plant growth regulator or with 0.25 mg/l BAP and/or 6% sucrose. Then, the regenerated shoots with 5 cm long were transferred to three-fourth strength MS without any plant growth regulator for root induction. All the above media contained 3% (w/v) sucrose and 8 g/l agar. The pH of the media was adjusted to 5.6 - 5.8 prior to the addition of agar and autoclaving at 121°C for 20 min. All the explants were cultured at $26 \pm 2^\circ\text{C}$ and under 16 hrs of day time with the light intensity of 2000 lux. Data on percentage of morphogenesis responses, such as callus and shoot formation and also number of shoots initiated per explants were recorded after 30 day.

The treatments were arranged in a completely randomized design (CRD) with three replicates per treatment and five explants per replicate. All data were subjected to arcsine in transformation. Data were analyzed statistically using SPSS ver. 13.0 software. The mean values of different treatments were compared using DMRT (at 5% level).

Results and Discussion

Callus formation was observed about ten days after the initiation of cotyledonary node explants culture and within two weeks of hypocotyl explants culture. No sign of callus growth was seen in leaf explants. The calli were creamy and light yellowish in color with soft structures (Fig. 1c). The analysis of variance indicated that the type and the concentration of plant growth regulators and remarkably the source of explants, made a significant difference in terms of the frequency of callus induction. The highest callus induction (100%) was observed from cotyledonary node explants on MS supplemented with 2, 4-D at 1 mg/l in combination with Kn at 0.5 mg/l (Table 1).

Different types of explants have been used for *in vitro* callus induction of many medicinally important plants. For example, leaf explants of *Tylophora indica* (Dennis and Philip 2005), leaf and nodal explants of *Cardiospermum halicacabum*

(Dennis and Maseena 2006), nodal cuttings of *Sarcostemma brevistigma* (Dennis and Shankar 2009) and hypocotyls of *Dorema ammoniacum* (Irvani et al. 2010). According to the results, the cotyledonary nodes were more effective for callus induction than hypocotyl explants and also the quality of calli.

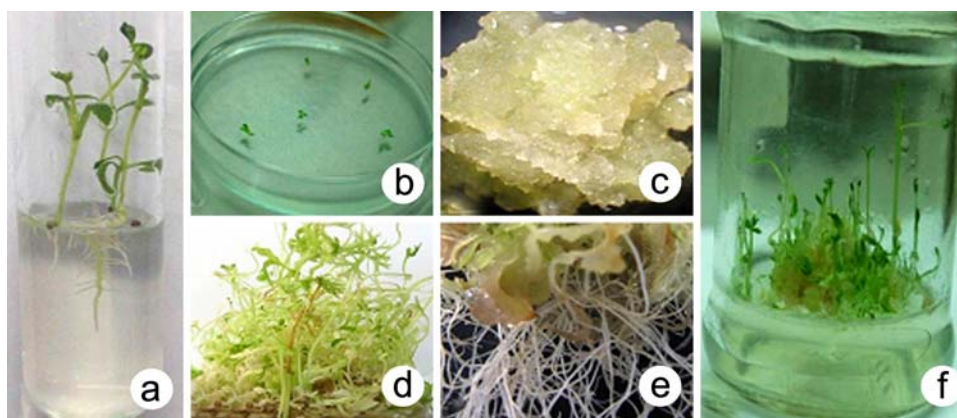


Fig. 1a. Forty days old seedlings of *T. copticum*. b. Leaf explants on callus induction medium. c. Creamy, yellowish calli with soft structures after 30 days of inoculation. d. Shoot regeneration from callus after 30 days of inoculation. e. Root regeneration after 60 days of inoculation and f. regenerated plantlet.

Table 1.a. Composition and concentrations of PGRs for callus induction from leaf, hypocotyl and cotyledonary node explants of *T. copticum*.

Plant regulators (mg/l)					Callus induction (%)		
Auxins		Cytokinins			Explant		
2,4-D	NAA	IAA	BAP	Kn	Leaf	Hypocotyl	Cotyledonary node
0.2	-	-	-	-	0 ^c	0 ^c	0 ^c
0.3	-	-	-	-	0 ^c	0 ^c	0 ^c
0.5	-	-	-	-	0 ^c	0 ^c	100 ^a
1	-	-	-	-	0 ^c	0 ^c	100 ^a
1	-	-	-	0.5	0 ^c	96 ^a	100 ^a
1	-	-	0.5	-	0 ^c	100 ^a	100 ^a
2	-	-	-	-	0 ^c	0 ^c	100 ^a
2	-	-	-	0.5	0 ^c	100 ^a	100 ^a
2	-	-	0.5	-	0 ^c	100 ^a	100 ^a
3	-	-	-	-	0 ^c	0 ^c	0 ^c
-	2	-	-	-	0 ^c	0 ^c	100 ^a
-	-	3	-	-	0 ^c	0 ^c	100 ^a
-	0.08	1	-	-	0 ^c	80 ^b	100 ^a

The induction of organic or embryogenic callus was usually promoted by an auxin, especially 2,4-D (Carman 1990, Bennici and Bruschi 1999, Kamo et al. 2005). Similar results of 2,4-D in callus induction were observed. Callus induction through an auxin-cytokinin combination has been reported for several plants including *Astragalus adsurgens* (Luo et al. 1999), lavandin (*Lavandula × intermedia* (Dronne et al. 1999), *Eucalyptus nitens* and *E. globules* (Bandyopadhyay et al. 1999), *Valeriana edulis* (Castillo et al. 2000), *Lathyrus sativus* (Zambre et al. 2002), *Diffenbachia* sp. (Shen et al. 2007) as well as the current study which is a combination of auxin (2,4-D) and cytokinin (BAP, Kn) induced organic calli.

Table 2. Effect of different composition and concentrations of PGRs BA on regeneration of adventitious *T. copticum* shoot from callus produced from hypocotyls and cotyledonary node explants.

Shoot induction treatments	Hypocotyl			Cotyledonary node		
	Shoots (%)	Mean no. of shoots	Shoots height (cm)	Shoots (%)	Mean no. of shoots	Shoots height (cm)
MS	94 ^{a-b}	1.2 ± 0.3 ^{b-d}	1.6 ± 1 ^{b-c}	100 ^a	3.5 ± 1.3 ^a	2.8 ± 0.8 ^a
1/2 MS	60 ^{c-d}	0.8 ± 0.4 ^{d-e}	0.9 ± 0.2 ^{d-g}	66 ^{b-d}	1 ± 0.3 ^{c-e}	1.1 ± 0.1 ^{c-f}
3/4 MS	27 ^e	0.3 ± 0.4 ^e	0.4 ± 0.6 ^{f-g}	94 ^{a-b}	1.5 ± 0.6 ^{b-d}	1.8 ± 0.7 ^b
MS + 0.25BAP	60 ^{c-d}	1 ± 0.6 ^{c-e}	0.8 ± 0.6 ^{d-g}	77 ^{a-c}	1.9 ± 0.7 ^{a-b}	1.3 ± 0.6 ^{b-d}
1/2 MS + 0.25 BAP	71 ^{a-d}	1.5 ± 0.7 ^{b-d}	0.9 ± 0.2 ^{d-g}	55 ^{c-d}	1.5 ± 0.9 ^{b-d}	0.8 ± 0.5 ^{d-g}
3/4 MS + 0.25 BAP	27 ^e	0.8 ± 0.5 ^{d-e}	0.3 ± 0.1 ^g	44 ^{d-e}	1.6 ± 0.7 ^{b-d}	0.5 ± 0.3 ^{e-g}
MS % 6 sucrose	77 ^{a-c}	1.4 ± 0.6 ^{b-d}	1.1 ± 0.1 ^{c-e}	72 ^{a-d}	1.1 ± 0.6 ^{b-d}	1.3 ± 0.1 ^{b-d}
1/2 MS % 6 sucrose	71 ^{a-d}	1.8 ± 1.3 ^{b-c}	1.1 ± 0.7 ^{c-f}	55 ^{c-d}	1.4 ± 0.4 ^{b-d}	0.7 ± 0.3 ^{d-g}
3/4 MS % 6 sucrose	66 ^{b-d}	1.4 ± 0.9 ^{b-d}	1 ± 1 ^{c-g}	71 ^{a-d}	1.7 ± 0.8 ^{b-c}	1.1 ± 0.6 ^{c-f}

Means within a column followed by the same letters are not significantly different by DMRT ($p \geq 0.05$).

The calli derived from various explants were subcultured on shoot induction media. One week after transferring to regeneration medium, embryogenic calli enlarged rapidly and green spots appeared on the surface making the whole callus brown by the 30 days. Green shoot buds started emerging on the various embryogenic calli on the 2nd weeks (Fig. 1d). The statistical analysis revealed that the regeneration frequency and the number of shoots per callus differed significantly in hypocotyls and cotyledonary cuttings derived from calli and also in different composition of the cultural medium. Selection of explant is one of the important steps for successful shoot regeneration (Koca and Aasim 2015). Interestingly, the calli derived from cotyledonary node segments showed significantly higher frequency of plantlet regeneration and number of plantlets than the calli derived from hypocotyl segments. The highest regeneration

frequency (100%) and maximum average number of shoots per explants (3.5 ± 1.3) were observed on free hormone MS (Table 2). Plantlets rooted on the three/fourth MS without any plant growth regulator with 75% efficiency after 30 days (Fig. 1e). After 60 days, the number and the length of roots were enough for transferring to the pot (Fig. 1f).

There are a few reports on tissue culture of *Trachyspermum* sp. or *Carum* sp. e.g. plant regeneration from callus of *T. ammi* (Jasrai et al. 1992, Sehgal and Abbas 1994), callus induction and regeneration from apical bud of *Carum copticum* L. (Salehi et al. 2014), direct shoot micropropagation system in *T. ammi* by using seed as explants (Koca and Aasim 2015), direct somatic embryogenesis from cotyledon and cotyledonary node explants in *T. ammi* (Purohit and Kothari 2007).

The present study on callus induction and regeneration of *T. copticum* may help conservation of the species and possibly will lead to the production of secondary metabolites and extraction of active compounds from callus sources.

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