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Direct shoot regeneration from hypocotyl explants of *Heracleum candicans* Wall: A vulnerable high value medicinal herb of Kashmir Himalaya

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Heracleum candicans **belongs to the family Apiaceae, and is categorized as a vulnerable Himalayan medicinal herb. Due to its diverse chemical constituents it is having an increased demand in pharmaceutical industries, especially in international market. This herb is commercially useful as a major source of Xanthotoxin which is widely used to treat leucoderma and to prepare suntan lotions. During the present study direct shoot regeneration has been achieved from hypocotyl explants on MS (Murashige and Skoog, 1962) medium fortified with different plant growth hormones. Shoot bud regeneration was achieved on media augmented with auxins like Indoleacetic acid (IAA), 2,4 - dichlorophenoxyacetic acid (2,4-D) and cytokinins like Kinetin (KN) and Benzyladenine (BA). Due to the presence of 2,4-D in the medium friable callus development has been reported in some cultures. Regenerated shoots were transferred to MS basal medium for root induction and later on successfully acclimatized in vermiculite under controlled conditions. This is the first report on plant regeneration from hypocotyl explants of *H. candicans* and could be used as an alternative for large scale propagation and conservation of this vulnerable plant species.**

Key words: Apiaceae, explants, callus, auxins, cytokinins, MS medium.

INTRODUCTION

Heracleum candicans Wall. Commonly known as Patrala, is a perennial herb endemic to the northwest Himalayas. It is found distributed in mountains and alpine zones of Bhutan, Afghanistan, south-west China, West Pakistan, Nepal and India at an altitude of 2000 to 4300 m asl. It is one amongst the rare Himalayan resources and the most valued species of genus *Heracleum* and produces optimum quantity of Xanthotoxin (Kaul, 1989). Almost each and every part of this plant has the ability to cure various diseases and its medicinal efficacy is well recognized by indigenous communities as well as modern systems of medicine (Rawat et al., 2013). In Himalayan region, its juvenile shoots and leaves are eaten mostly by shepherds, and also used as fodder for

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increasing the milk production of cows (Badola and Butola, 2005). The native people use its roots for treating various skin diseases like eczema and itches (Gaur, 1999). The roots contain 0.1% essential oil and bergapten. Its roots also yield xanthotoxin which is used to treat leucoderma and in preparation of suntan lotions (Kaul, 1989). Its fruits are used as an aphrodisiac and nerve tonic (Satyavati et al., 1987). Activity-guided isolation has also shown heraclenin to be the anti-inflammatory principle present in *H. candicans.* The plant possesses potent stimulatory effect on melanogenesis with significant enhancement of cell proliferation (Matsuda et al., 2005). Extracts of root and shoot showed antibacterial activity (Kaur et al., 2005). Twenty-eight compounds were isolated and identified from essential oil using gas chromatography–flame ionization detection and GC–mass spectrometry (GC–MS) analysis (Chauhan et al., 2014). These compounds are very useful for the pharmaceutical, flavor and fragrance industries. This plant species propagates by seed, but seed viability is very low (Butola and Badola, 2004). This poor seed germination together with overharvesting of plants from natural habitats for commercial utilization puts this plant species under severe threat. Owing to its unsustainable harvesting in nature, this plant species is categorized as endangered for northwestern Himalayas (Anonymous, 1998; CAMP, 2003), and vulnerable for the state of Himachal Pradesh and Jammu and Kashmir (Ved et al., 2003). Only a few reports are available on *in vitro* plant regeneration from leaf, petiole and shoot tip explants of *H. candicans* (Xing, 2006; Sharma and Wakhlu, 2001; Sharma and Wakhlu, 2003) and to date, there are no reports on plant regeneration from hypocotyl explants of *H. candicans.* This study describes a rapid protocol for direct shoot regeneration from hypocotyl explants of *H. candicans.*

MATERIALS AND METHODS

Raw material

Seeds were obtained from the plants growing in Kashmir university botanical garden and washed under running tap water for about 30 min. Detergent Labolene 1% v/v (LOBA Chemie- Laboratory reagents and fine chemicals) containing few drops of surfactant, Tween 20 (Hi Media) were then added to the seeds for washing. This was followed by washing with tap water to remove the detergent and finally washed with double distilled water under laminar air flow cabinet. Finally the seeds were sterilised with 2% sodium hypochlorite solution (Hi Media) for 30 min. After 30 min disinfectant solution was decanted and the seeds were washed 5-6 times with double distilled water so as to remove any traces of the sterilant. The sterilized seeds were then transferred on to petriplates filled with sterilized cotton.

Implementation of plant propagation protocol

Hypocotyl explants were taken from seedlings and cultured on MS medium (Hi Media) containing auxins, cytokinins and auxin-cytokinin combinations. The cultures were maintained at a temperature of 25±2°C, light intensity of 3000 lux and a regular photoperiod of 16 h. Each experiment was repeated at least twice and data was analysed by calculating Standard Error (SE) of various treatments and means were analyzed by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The hypocotyl explants inoculated on MS medium without added growth hormones showed no response for shoot bud formation. However an interplay of auxin and cytokinin individually and in combinations showed enhanced shoot bud proliferation. Adventitious shoot buds formed from hypocotyl explants directly within 15-30 days without an intervening callus phase. Among auxins 2,4-D showed shoot bud proliferation with best response obtained on 2,4-D 3 mg/l (Figure 1). After 2,4-D 3 mg/l the number of shoots per explant declined (Table 1). Among cytokinins, BAP was more effective as compared to Kn in shoot bud induction (Table 2). The best response, however, was obtained at BAP 3 mg/l (Figure 2a) where an average of 4.5 shoots formed per explant within 20 days of inoculation onto the medium. Number of shoots formed per explant declined after BAP 3 mg/l. When explants were inoculated on MS medium augmented with Kn, there occurs a gradual increase in the number of shoots upto 4 mg/l (Figure 2b) which is directly proportional to increasing Kn concentration. After 4 mg/l there occurs a decline in the number of shoots per explant. Among the auxin and cytokinin combinations used, BAP 3 mg/l and IAA 2 mg/l (Figure 3) gave best results with an average of 9.4 shoots per explant, being much higher than that obtained when BAP, Kn and 2,4-D were used individually (Table 3). Roots were obtained when regenerated shoots were transferred to MS basal medium within a period of 10 days (Figure 4a). Rooted plantlets were transferred to pots containing vermiculite and were successfully acclimatized under green house (Figure 4b).

In vitro regeneration of plants is influenced by many factors such as environment around cultures, media composition, source of explant, plant growth hormones and genotype (Zhang et al., 1998; Bano et al., 2010; Jana and Shekhawat, 2011; Dhir and Shekhawat, 2014). The results obtained in the present study indicate clearly that high frequency direct shoot regeneration is achieved from hypocotyl explants of *H. candicans* on MS medium supplemented with BAP 3 mg/l + IAA 2 mg/l. Similar results were obtained in *Cuminum cyminum* L. were hypocotyl was also reported as the most responsive explant in terms of shoot organogenesis (Tawfik and Noga, 2002). Hypocotyl explant was also used for shoot proliferation in *Ferula assa-foetida* L. on MS medium supplemented with auxin cytokinin combinations (Zare et al., 2010). It has also been reported that in *Dorema ammoniacum* D. Don. BAP alone or a combination of

Figure 1. Shoot regeneration: MS+2,4-D 3 mg/l.

Table 1. Effect of Auxins on multiple shoot formation from hypocotyl explant.

Treatments	Mean number of shoots±SE	Mean height of shoots(cm)±SE	Mean Number of Days	% Culture response
MS basal	-	-	-	-
2,4-D 1 mg/l	1.0±0.2^{a}	1.9±0.1^{a}	32	30
2,4-D 2 mg/l	1.9±0.3^{a}	1.8±0.2^{a}	29	50
2,4-D 3 mg/l	3.2±0.2^{b}	2.9±0.2^{b}	23	60
2,4-D 4 mg/l	1.7±0.2^{a}	1.8±0.1^{a}	27	40
2,4-D 5 mg/l	1.6±0.2^{a}	1.4±0.1^{a}	30	30

(10 replicates per treatment).*Means followed by different superscripts are significantly different from each other at 5% level.

Table 2. Effect of Cytokinins on multiple shoot formation from hypocotyl explant.

Treatments	Mean number of shoots±SE	Mean height of shoots(cm)±SE	Mean Number of Days	% Culture response
MS basal	-	-	-	-
BAP 1 mg/l	-	-	-	-
BAP 2 mg/l	2.6±0.3^{ab}	2.1±0.1^{a}	28	70
BAP 3 mg/l	4.5±0.3^{c}	4.1±0.1^{b}	20	100
BAP 4 mg/l	2.9±0.2^{b}	2.5±0.3^{a}	29	80
BAP 5 mg/l	1.7±0.2^{a}	2.3±0.1^{a}	30	40
Kn 1 mg/l	1.7 ±0.2^{a}	2.5±0.1^{a}	32	60
Kn 2 mg/l	1.9±0.2^{a}	2.8±0.2^{a}	28	70
Kn 3 mg/l	2.8±0.4^{a}	2.9±0.2^{a}	26	80
Kn 4 mg/l	4.2±0.4^{b}	4.1±0.3^{b}	23	90
Kn 5 mg/l	2.5±0.4^{a}	2.8±0.3^{a}	29	40

(10 replicates per treatment), *Means followed by different superscripts are significantly different from each other at 5% level.

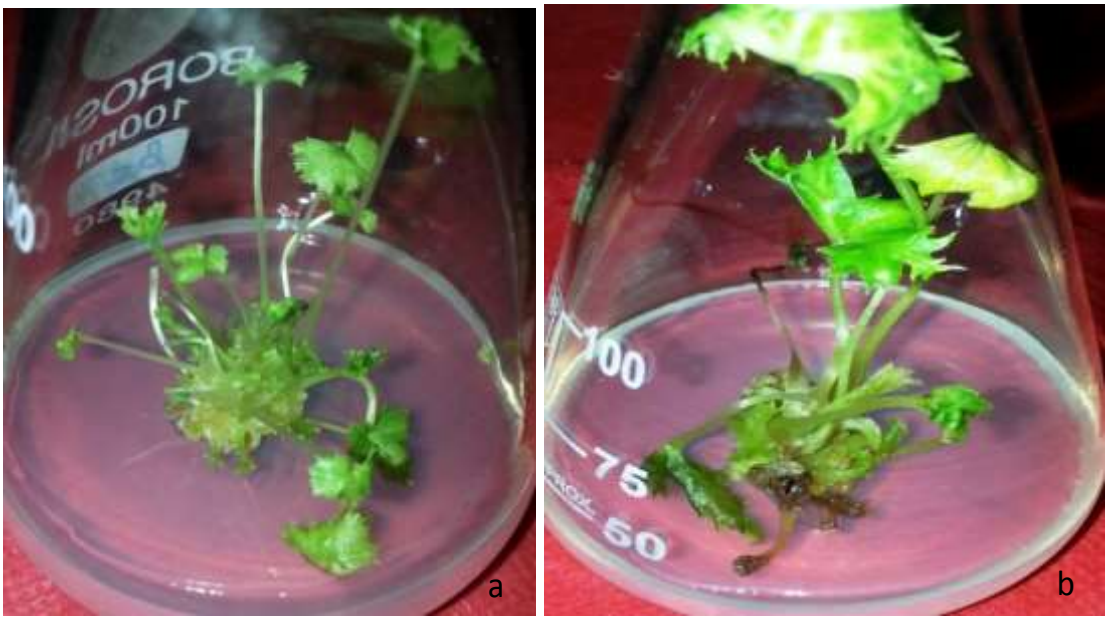

Figure 2. Shoot regeneration: a) MS+ BAP 3 mg/l, b) MS + Kinetin 4 mg/l.

Figure 3. Shoot regeneration: MS + BAP 3 mg/l + IAA 1 mg/l.

auxins proved effective for shoot regeneration (Irvani et al., 2010). Direct adventitious shoot bud formation from hypocotyl explant was also obtained in *Millettia pinnata* (L.) on MS medium supplemented with 8.88 µM BAP (Nagar et al., 2017). Results obtained for rooting were in accordance with the results obtained in *Daucus carota* where in the regenerated shoots showed best root regeneration on MS medium without any added growth

Table 3. Effect of Auxin cytokinin combinations on multiple shoot formation from hypocotyl explant.

Treatments	Mean number of shoots±SE	Mean height of shoots(cm)±SE	Mean Number of Days	% Culture response
MS basal	-	-	-	-
BAP 3 mg/l + IAA 1 mg/l	6.9±0.5[a]	2.9±0.3[a]	22	70
BAP 3 mg/l + IAA 2 mg/l	9.4±0.8[b]	5.0±0.4[b]	15	100
BAP 3 mg/l + IAA 3 mg/l	6.7±0.4[a]	2.5±0.2[a]	25	40

(10 replicates per treatment), *Means followed by different superscripts are significantly different from each other at 5% level.

Figure 4. (a) Root regeneration, (b) Acclimatization of regenerated shoots.

regulators.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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