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Effect of explant source and different hormonal combinations on *in vitro* regeneration of *Heracleum candicans* Wall: An important medicinal herb

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***In vitro* regeneration of plants is influenced to a great extent by genotype, the type of explant used, media composition and plant growth regulators. This research was devised to study the effect of explant source and different hormonal combinations on the *in vitro* regeneration of *Heracleum candicans.* Two different explants viz; leaf and petiole were used to investigate their morphogenic response on MS medium supplemented with different concentrations of auxins (2, 4-dichlorophenoxyacetic acid and indole 3-acetic acid) and cytokinins (6-benzyl amino purine and kinetin) either individually or in combinations. Petiole explants were most responsive for callus production as well as shoot regeneration. 2,4-D at 3 mg/L proved to be the best concentration for callus induction. For shoot regeneration, MS medium supplemented with BAP at 3 mg/l and IAA at 2 mg/l proved to be effective in production of 21±0.7 mean number of shoots in 90% of cultures. Regenerated shoots were separated and rooted on full strength MS basal medium producing 6.9 average numbers of roots per shoot in 90% of cultures. The present research yielded a suitable explant and optimum concentrations of different plant growth regulators for *in vitro* regeneration of this important medicinal herb.**

Key words: *Heracleum candicans,* leaf, petiole, callus, shoot regeneration.

INTRODUCTION

Heracleum candicans is a perennial herb endemic to the northwest Himalayas and is found growing in alpine zones of West Pakistan, Nepal, Bhutan, Afghanistan and India (Sharma and Wakhlu, 2001). Propagation occurs by means of seeds, however, the seed germination is poor. This poor germination coupled with unsustainable harvesting of plants from natural habitats for commercial utilization threatens the existence of this plant species (Kaul, 1989). Being the main source of xanthotoxin, it has an immense demand in pharmaceutical industries (BCIL, 1996). Xanthotoxin is widely used to treat leucoderma and to prepare suntan lotions (Kaul, 1989). The fruit is used as an aphrodisiac and nerve tonic (Satyavati and Gupta, 1987). Activity-guided isolation has also shown

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heraclenin to be the anti-inflammatory principle present in *H. candicans.* Extracts of root and shoot showed antibacterial activity (Kaur et al., 2006). A lengthier vegetative growing phase coupled with low seed viability in this species (Butola and Badola, 2004, 2006) offer fewer chances to revive and shift into fresh habitats. Because of the high market price of its rootstock, it is one of the important medicinal plants exported from India (BCIL, 1996). Owing to its unsustainable harvesting in nature, this plant is categorized as endangered for northwest Himalayas (Anonymous, 1998; CAMP, 2003), and vulnerable for the Jammu & Kashmir State in India (Ved et al., 2003).

The instantaneous rising demand of herbal medicine is creating heavy pressure on some selected important medicinal plants in the wild due to overexploitation. Several of these medicinal plant species have slow growth rates, low population densities and narrow geographic ranges (Kala, 2009); therefore, they are more likely to undergo extinction (Anandanayaki, 2010). The vanishing of such dynamic and huge amounts of biodiversity presents one of the serious challenges for the world community to halt the devastation of plant diversity that is essential to meet the present and future needs of mankind. To regenerate organs from plant tissues, media components and hormone concentrations can be optimized. Tissue culture is not only a necessary enabling technology for transgenic plant production but is also used for *in vitro* propagation of valuable plants. Large-scale propagation is a prerequisite to meet the pharmaceutical needs, and also for effective conservation of endangered plant species. Tissue culture, from conservation point of view is important as it requires a small amount of propagules for mass multiplication providing huge quantity of raw material to herbal drug industry. Micropropagation of plant species has been found to be explant based. In case of Apiaceae family, different explants namely intermodal segments (Yoshimatsu and Shimomura, 1991), shoot tips (Wakhlu and Sharma, 1998; Rachetti and Biradar, 2016; Srilakshmi et al*.,* 2016), single-node explants (Chandrika et al., 2015; Tuncer, 2017), hypocotyl explant (Thapa et al*.*, 2015; Mandal and Sharma, 2016), leaf explant (Rao et al*.,* 2015; Soorni and Kahrizi, 2015) and petiole explant (Askari-Khorasgani et al*.*, 2013; Torabi et al*.,* 2014; Sharma, 2015) have been used for *in vitro* regeneration of different plants. The present study is aimed at identifying the best type of explant as well as the most efficient growth regulator concentration and combinations for shoot formation and regeneration of *H. candicans.*

MATERIALS AND METHODS

For standardization of *in vitro* regeneration protocols, two different explants viz; leaf and petiole were used. Leaf and petiole explants obtained from Kashmir University Botanical Garden (Voucher Specimen Number 2616-(Ref.No.F/Herbarium-Specimen COPT) KU/2017) were firstly washed with tap water in order to remove dust, dirt and other undesirable materials followed by washing with detergent solution (Labolene) containing 4 drops of Tween 20. This was followed by washing with tap water to eliminate the detergent. The explants were washed 2 to 3 times with double distilled water in a laminar air flow cabinet. Finally, the explants were sterilized with 2% sodium hypochlorite solution for different time durations. The surface disinfected explants were then washed 3 to 4 times with autoclaved double distilled water to remove the last traces of the sterilants. The disinfected plant material was then put into pre-autoclaved petri-dishes, cut into appropriate size and finally aseptically cultured on the medium. Each treatment involved about 10 to 30 explants and each experiment was repeated twice. Explants were inoculated onto MS basal medium (control) and MS medium fortified with different plant growth regulators both individually and in combinations. Throughout the experiments, culture room was kept entirely germ-free and explants were cultured under cool white light with 1500-3000 Lux light intensity. The temperature of the room was maintained between 22±4°C with 60-70% relative humidity and photoperiod of 16 h.

The cultures were regularly observed, changes in explant were recorded on weekly basis and the data was put into a suitable arrangement in tabulated form. The parameters recorded were induction of callus, texture of callus, shoot regeneration and root regeneration. Each experiment was repeated twice, data was analyzed by calculating Standard Error (SE) of various treatments and means were analyzed by analysis of variance (ANOVA).

RESULTS

During the present study, the comparative effect of two different explants viz. leaf and petiole and concentrations of auxins and cytokinins on the morphogenesis of *H. candicans* was analyzed. The results are summarized in Tables 1 to 4. The data revealed that there were significant differences in the type of explant used and the effect of different concentrations and combinations of 2,4-D, IAA, BAP and Kn.

Evaluation of the effect of explant type and hormonal concentration on callus induction

During the present investigation, callus induction from leaf and petiole explant was obtained on MS medium supplemented with auxins both individually and in combination with cytokinins. Among auxins, five different concentrations of 2,4-D were used for callus induction. Callus induction was observed in both the explants and callus induction percentage varied significantly among the two explants. The 2,4-D at 3 mg/L was the optimum or threshold concentration for callus induction in both the explants, however the number of days taken for callus induction were shortest in the case of petiole explants. In order to examine the effect of auxin-cytokinin combinations on callus induction, leaf and petiole explants were inoculated on MS medium supplemented with 2,4-D (3 mg/L) in combination with five different concentrations of Kn. The 2,4-D at 3 mg/L and Kn at 2 mg/L proved to be the optimal concentration for maximum callus induction, with 100% culture response occurring in the petiole explant. By increasing the

Table 1. Effect of explant type and hormonal concentration on callus induction.

Explant	2,4-D concentration (mg/L)	Initiation of callus (days)	% Culture response
Leaf	1	38	50
	2	33	70
	3	30	90
	4	40	30
	5	0	0
Petiole	1	21	40
	2	19	80
	3	12	100
	4	20	70
	5	25	60

Table 2. Effect of explant type and auxin-cytokinin combinations on callus induction.

Explant	2,4-D concentration mg/L	Kn concentration (mg/L)	Initiation of callus (days)	% Culture response
Leaf	**3**	1	24	40
	3	2	20	90
	3	3	25	80
	3	4	27	70
	3	5	29	60
Petiole	**3**	1	0	0
	3	2	14	100
	3	3	25	80
	3	4	28	50
	3	5	30	30

Table 3. Effect of explant type and hormonal concentration on shoot regeneration.

Explant	BAP concentration (mg/L)	Average number of shoots±SE	Average height of shoots (cm)±SE	Initiation of callus (days)	% Culture response
Leaf	1	0	0	0	0
	2	3 ± 0.2^{a}	2.6 ± 0.1^{a}	18	70
	3	5.9 ± 0.5^{c}	5.7 ± 0.2^{c}	15	80
	4	4.6 ± 0.3^{bc}	4.0 ± 0.2^{b}	21	50
	5	3.6 ± 0.3^{ab}	2.1 ± 0.2^{a}	23	30
Petiole	1	3.1 ± 0.3^{a}	1.3 ± 0.0^{a}	21	30
	2	9.2 ± 0.5^{ab}	5.8 ± 0.1^{ab}	18	60
	3	15.2 ± 0.6^{b}	7.6 ± 0.2^{c}	16	100
	4	6.7 ± 0.2^{a}	4.4 ± 0.1^{bc}	20	50
	5	2.2 ± 0.2^{a}	1.5 ± 0.1^{ab}	23	30

Different letters on the values indicate that the means are significantly ($P < 0.05$) different (Tukey's HSD test).

concentration of Kn further, there occurs a continuing decrease in callus induction, because of the fact that increasing the ratio of cytokinin to auxin favors shoot regeneration.

Table 4. Effect of explant type and auxin-cytokinin combinations on shoot regeneration.

Explant	BAP concentration (mg/L)	IAA concentration (mg/L)	Average number of shoots±SE	Average height of shoots (cm)±SE	Initiation of callus (days)	% Culture response
Leaf	3	1	9.3±0.7 ^{cd}	6.3±0.1^{bc}	15	70
	3	2	11.2±0.6 ^{d}	6.8±0.1^{c}	10	80
	3	3	7.5±0.4 ^{bc}	6.0±0.1^{bc}	16	60
	3	4	6.3±0.4 ^{b}	5.4±0.3^{b}	19	50
	3	5	3.2±0.2 ^{a}	1.4±0.1^{a}	21	30
Petiole	3	1	11.3±0.5^{c}	3.9±0.1^{c}	14	60
	3	2	21±0.7^{d}	4.9±0.1^{a}	11	100
	3	3	6.6±0.6^{b}	3.5±0.2 ^{bc}	19	70
	3	4	3.8±0.2^{a}	3.0±0.1^{b}	24	40
	3	5	3.1±0.3^{a}	2.2±0.0^{a}	28	30

Different letters on the values indicate that the means are significantly ($P < 0.05$) different (Tukey's HSD test).

Evaluation of the effect of explant type and hormonal concentration on shoot regeneration

During the present study, shoot regeneration was witnessed from both the explant types on MS medium supplemented with cytokinins individually and in combination with auxins. When MS medium was fortified, five different concentrations of BAP individually, BAP at 3 mg/L proved to be best medium for shoot regeneration with maximum number of shoots obtained from petiole explant in minimum number of days. Among five different auxin and cytokinin combinations used, BAP at 3 mg/L and IAA at 2 mg/L gave best results in both the explant types with maximum number of shoots regenerated from petiole derived calli. By increasing the auxin concentration from 2 to 5 mg/L in the medium, a significant decrease occurred in the frequency and number of shoots produced per explant with an increase in the amount of callus. For root formation, the *in vitro* raised shoots were subcultured on MS basal medium and MS medium containing IAA, NAA and IBA individually. In the present study, MS basal medium was effective for root regeneration. Among hormone supplemented medium, the most effective concentration at which maximum root induction was achieved was IAA at 2 mg/L.

DISCUSSION

The explant source is a vital factor for *in vitro* growth and development of plant species affecting callus production, shoot bud induction as well as regeneration and multiplication. During the present research, statistical analysis revealed that there were significant differences between the two explant types used. Petiole explant proved to be the most effective explant for both callus induction as well as shoot formation (Figure 1). Patra et al. (1998) developed a successful protocol for *in vitro* plant regeneration from callus derived from leaf explants in *Centella asiatica* (L.). Mohapatra et al*.* (2008) also performed *in vitro* studies in *C. asiatica* using leaf explant. The study revealed that frequency of multiple shoot regeneration was much higher on MS medium supplemented with combination of auxin and cytokinin than cytokinins used individually. Sharma and Wakhlu (2001) obtained similar results for adventitious shoot induction from petiole explants of *H. candicans* Wall. However, the researchers used 2,4-D rather than IAA used in the present study. Makunga et al*.* (2003) obtained direct shoot regeneration from petiole explant of *Thapsia garganica* on MS medium supplemented with 0.5 mg /L NAA and 1.5 mg/l BAP which is in contrast with our results. Sharma (2009) achieved callus initiation from petiole explants of *H. candicans* on MS medium supplemented with 0.5 mg/L 2,4-D and 0.5 mg/L BAP. In addition, the best combination of growth regulators for maximum callus growth was obtained upon subculture of callus to a medium supplemented with 1 mg/L 2,4-D and 0.25 mg/L Kn. Askari-Khorasgani et al*.* (2013) also used petiole explant for direct regeneration of *Kelussia odoratissima.* The maximum rate of shoot multiplication was achieved by inoculation of petiole explants on MS medium fortified with 2 mg/L BAP and 0.1 mg/L NAA. Sharma (2015) performed studies on callus induction from petiole explants in *Ferula jaeschakeana* in which explants were cultured on MS medium supplemented with 2,4-D for callus initiation. The best results of callus induction were obtained on medium fortified with 1 mg/L 2,4-D. The addition of Kn in combination with 2,4-D enhanced the callus formation from the explants.

Conclusion

The present study yielded a suitable explant and

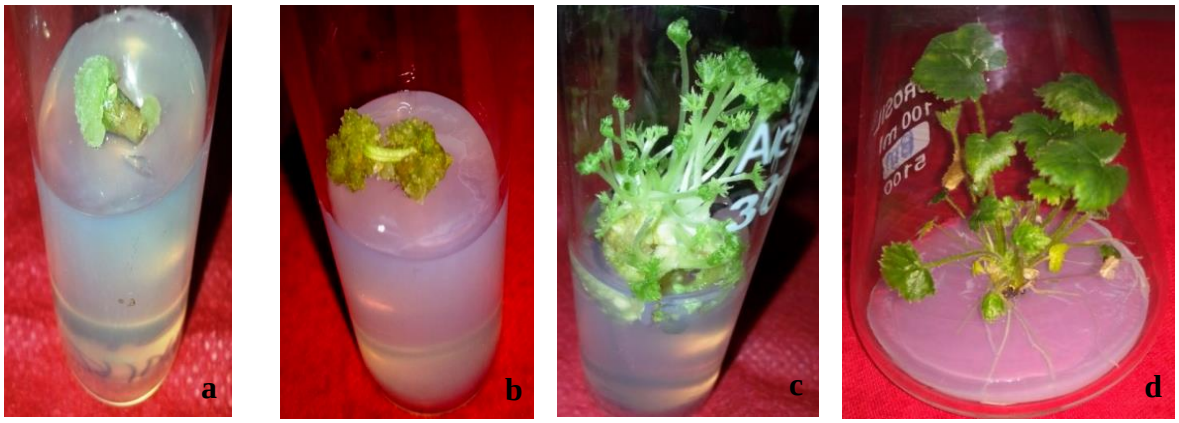

Figure 1. (a) and (b) callus induction from petiole explant (c) Shoot regeneration from petiole derived calli (d) Root regeneration.

optimum concentrations of different plant growth regulators for *in vitro* regeneration of this important medicinal herb.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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