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Comparative Effect of Different Phytohormones on Micropropagation of *Heracleum candicans Wall*- An Important Medicinal Plant

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A B S T R A C T

Heracleum candicans is a rare Himalayan medicinal herb valued for its production of optimum quantity of Xanthotoxin. Xanthotoxin isolated from roots is widely used to treat leucoderma and to prepare suntan lotions. The overall goal of the present research was to establish the most suitable phtyohormone for *in vitro* regeneration of *Heracleum candicans*. During the present study, cotyledon explants were inoculated on MS basal medium supplemented with various plant growth regulators including auxins (2, 4 Dichlorophenoxyacetic acid, Indole 3-Acetic Acid, Indole 3-Butyric Acid and α-Naphthalene Acetic Acid) and cytokinins (6-Benzyl Amino Purine and Kinetin) to find the effect of these growth regulators in inducing differentiation and re-differentiation in these explants. Among different concentrations and combinations of phytohormones, 2, 4 Dichlorophenoxyacetic acid at a concentration of 3 mg/l was effective in producing light green and fragile callus in 100% cultures. For shoot regeneration, 6-Benzyl Amino Purine 3 mg/l and Indole 3-Acetic Acid 1 mg/l proved most effective in 100% cultures. For root induction, full strength MS basal proved to be the best medium producing 6.9±0.3 average number of roots per shoot within a period of 15 days in 90% of cultures.

***Key words*:** Callus, *Heracleum candicans,* Phytohormones, Regeneration, Xanthotoxin

Heracleum belongs to family Apiaceae which is one of the most important families of flowering plants with more than 300 genera and 3000 species [1]. *Heracleum candicans* is an important medicinal plant. Almost every part of this plant has distinctive properties to cure various ailments and its medicinal worth is well recognized by ethnic communities as well as modern systems of medicine [2]. The plant is also used for curing different diseases such as phlegm and wind disorders, earache, stomach disorders, infection, bleeding, leprosy, fever due to wounds, and blood pressure [3]. The natives use its roots for treating skin diseases, eczema and itches [4]. Chemical investigation of the plant has revealed the presence of furocoumarins, namely, bergapten, heraclenol, xanthotoxin, phellopterin, angelicin, imperatorin, xanthotoxol, heraclenin, candibirin [5] and 8-gernoxypsoralen. Its roots yield xanthotoxin which is widely used to treat leucoderma and to formulate suntan lotions [6]. The fruit is used as an aphrodisiac and nerve tonic [7]. Heraclenin isolated from the roots of the plant has shown anti-inflammatory properties.

Tissue culture has arisen as an important method for safeguarding of threatened species [8-9]. Micropropagation protocols for cloning a variety of medicinal and aromatic plants have been developed over the years [10-12]. Micropropagation provides rapid, year-round production of new plants from minimal tissue samples. During the last three decades, various medicinal plants have been successfully propagated and re-established by means of media optimization and supplementation of plant growth regulators [13-15]. To obtain desired responses in plant tissue culture, plant growth regulators and their concentrations have to be carefully selected. During the present study, the hormonal requirements for the establishment of various explants leading to the development of complete *in vitro* raised plantlets varied a great deal. These hormones were used individually as well as in different combinations so as to develop efficient micropropagation protocols for its regeneration.

MATERIALS AND METHODS

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Seeds obtained from the plants growing in Kashmir university botanical garden were first rinsed thoroughly under tap water for half an hour. These were then washed with a detergent solution Labolene 1% v/v (LOBA Chemicals) containing few drops of surfactant, Tween 20 (1% v/v) (Hi Media). This was followed by washing with tap water to eradicate the detergent and lastly washed 2-3 times with double distilled water underneath laminar air flow hood. The seeds were finally sterilized with 2% sodium hypochlorite solution (Hi Media) for half an hour. After half an hour disinfectant solution was decanted and the seeds were washed 5-6 times with double distilled water so as to eradicate any traces of the sterilant. The disinfected seeds were then aseptically transferred on to Petri plates filled with sterilized cotton. Cotyledon explants cut out from aseptically germinated seedlings were used for callus induction and shoot regeneration. For culturing, sterilized MS medium (Hi Media) with pH 5.6 having 30g/l sucrose added with different concentrations and combinations of auxins and cytokinins were used. The cultures were retained at 25± 2°C, light intensity of 3000 lux and a regular photoperiod of 16 hrs. Well excised cotyledons were inoculated on MS medium fortified with varying concentrations of different phytohormones for callus induction and shoot regeneration. Two different auxins (2,4-D and IAA) and cytokinins (BAP and Kn) were used individually and in combinations in a concentration range of 1-5 mg/l. The cultures were daily examined for contamination and growth. The changes in explant were recorded on weekly basis and the data was put in proper arrangement and in tabulated form. The parameters recorded were induction of callus, texture of callus and shoot regeneration. Each experiment was repeated twice and data was analyzed by calculating Standard Error (SE) of various treatments and means were analyzed by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

During the present study, comparative effect of different concentrations of auxins and cytokinins on regeneration of *Heracleum candicans* was analysed. The results are summarized in (Table 1-2). The data revealed that there were significant differences in the effect of different concentrations and combinations of 2,4-D, IAA, BAP and Kn.

Table 1 Effect of different phytohormones on callus induction from cotyledon explant

Treatments			Mean number of days	Culture response
2,4-D (mg/l)	BAP (mg/l)	Kn (mg/l)		
1	0	0	0	0
2	0	0	22	50%
3	0	0	18	100%
4	0	0	14	80%
5	0	0	25	70%
0	1	0	31	40%
0	2	0	27	50%
0	3	0	13	90%
0	4	0	16	80%
0	5	0	19	60%
3	0	1	27	40%
3	0	2	22	60%
3	0	3	15	100%
3	0	4	19	70%
3	0	5	0	0

Table 2 Effect of different phytohormones on shoot regeneration from cotyledon explant

Treatments		Mean number of shoots ± SE	Mean height of shoots(cm) ± SE	Mean number of days	Culture response
BAP (mg/l)	IAA (mg/l)				
1	0	0	0	0	0
2	0	1.6±0.2[a]	1.5±0.1 [a]	22	60%
3	0	3.1±0.3 [c]	2.8±0.2 [b]	16	100%
4	0	1.8±0.2 [a]	1.6±0.1 [a]	24	80%
5	0	1.7±0.2 [a]	1.7±0.1 [a]	28	40%
1	1	2.4±0.3[b]	2.2±0.1 [b]	29	40%
2	1	1.9±0.3 [a]	1.9±0.1 [a]	15	80%
3	1	3.7±0.3[c]	3.4±0.3[c]	10	100%
4	1	1.9±0.2 [a]	2.6±0.2[b]	19	70%
5	1	2.3±0.3 [b]	2.3±0.1 [b]	20	50%

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

Effect of phytohormones on callus induction

Callus induction from cotyledon explant was attained on MS medium augmented with auxins and cytokinins both individually and in different combinations. Among auxins, 2,4-D at a concentration of 3 mg/l was effective in producing light green and fragile callus in 100% cultures within 14 days (Fig 1a). After 2,4-D 3mg/l, there occurs gradual decrease in callus induction. Among cytokinins used, MS medium fortified with BAP 3mg/l proved to be effective for callus induction in 90% cultures within 13 days. After BAP 3mg/l, there occurs a significant decrease in callus induction in cultures. Among auxin-cytokinin combinations, MS medium augmented with 2,4-D 3 mg/l and Kn 3mg/l proved to be the best concentration for callus

induction producing creamish and compact callus in 100% cultures within 15 days of inoculation. By increasing the concentration of Kn further, there occurs a gradual decrease in callus induction, because of the fact that increasing the ratio of cytokinin to auxin favours shoot regeneration.

Effect of phytohormones on shoot regeneration

Shoot regeneration was obtained after sub-culturing callus on MS medium fortified with cytokinins both individually and in combination with auxins. Among cytokinins, MS medium fortified with BAP 3 mg/l proved effective for shoot regeneration in 100% of cultures within 16 days. Number of shoots regenerated per explant reduced significantly by increasing the concentration of BAP from 3 mg/l to 5 mg/l. The study revealed that BAP 3 mg/l proved to be the threshold concentration for shoot regeneration. Among the auxin and cytokinin combinations used, BAP 3 mg/l and IAA 1 mg/l gave best results with an average of 3.7±0.3 shoots per explant in 100% cultures within a period of 10 days being higher than that obtained when BAP was used individually (Fig 1b).

(a) Callus induction on MS medium + 2,4-D 3 mg/l

(b) Shoot regeneration on MS medium + 2,4-D 3 mg/l + IAA 1 mg/l

(c) Root induction on MS basal medium

Fig 1 Different stages of micropropagation

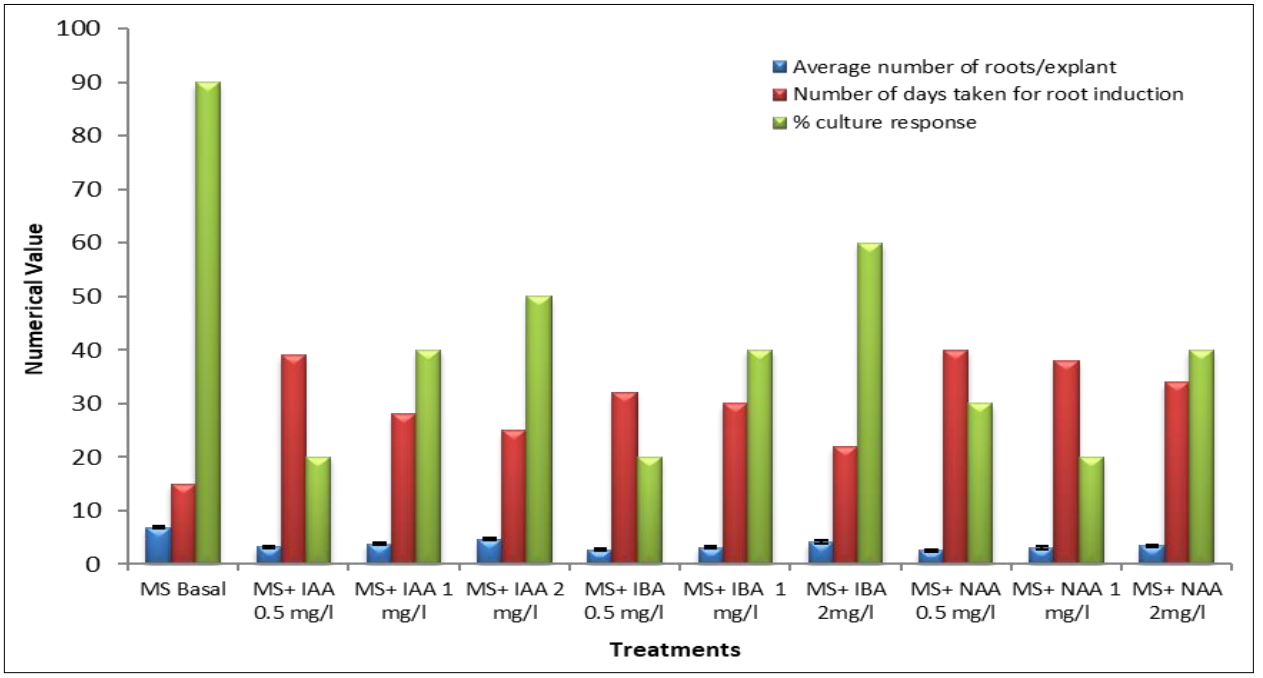

Fig 2 Effect of different phytohormones on root induction of *in vitro* raised shoots of *Heracleum candicans*

Effect of phytohormones on root induction

For root revival, the *in vitro* raised shoots were inoculated on MS basal medium and medium having auxins like IAA, NAA and IBA individually. MS basal proved to be the best medium producing 6.9±0.3 average number of roots per shoot within a period of 15 days (Fig.1c). Among hormone supplemented medium, the most effective concentration at which maximum root initiation was

achieved was medium augmented with IAA 2 mg/l by producing 4.7±0.3 average number of roots per shoot within a period of 25 days (Fig 2).

The results acquired during the present study are in agreement with the results obtained by Irvani *et al.* [16] in *Dorema ammoniacum* D. Don. As per their results callus was obtained from cotyledon explants on MS medium supplemented with 2,4-D alone and in combination with BAP or Kn. They also obtained shoot regeneration by sub-culturing callus on medium supplemented with BAP alone and in combination with auxins. Cotyledon explant was also used for callus induction and shoot regeneration in *Bunium persicum* [17]. Their results also show that callus induction from cotyledon explant was obtained on MS medium containing 2,4-D, BAP alone and 2,4-D in combination with Kn. Zare *et al.* [18] carried out similar studies in *Ferula assa-foetida* L. using cotyledon as explant for callus induction and shoot regeneration. Cotyledon explants developed nodular, green, organogenic and compact callus on MS medium augmented with 2,4-D and BAP alone and 2,4-D in combination with Kn. Shoot regeneration were obtained on medium supplemented with auxin-cytokinin combinations. However, progressively increasing the auxin concentration in the medium leads to decrease in the frequency and number of shoots with an increase in the amount of callus. Results attained 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 regulators. Singh *et al.* [19] achieved root induction in *Centella asiatica* on MS medium fortified with 1 mg/l NAA + 1 mg/l IBA.

CONCLUSION

During the present study growth regulators viz., auxins (IAA, IBA, NAA and 2,4-D) and cytokinins (BAP and Kn) were found to be responsible for inducing de-differentiation and re-differentiation in mature and differentiated tissues of cotyledon explants of *Heracleum candicans.* The present study revealed that 2,4-D proved to be best growth regulator either alone or in combination with Kn for callus induction. Experiments carried out for regeneration and multiplication of shoots revealed that BAP in combination with IAA proved to be most effective for producing healthy shoots.

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