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ADVENTITIOUS SHOOT REGENERATION FROM PETIOLE EXPLANTS OF *HERACLEUM CANDICANS* WALL.

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Summary

A method for adventitious shoot induction from petiole explants of *Heracleum candicans* is reported. Shoot buds were induced on Murashige and Skoog (MS) medium with 4.4 μ*M* 6-benzylaminopurine (BA) and 1.1 μ*M* 2,4-dichlorophenoxyacetic acid (2,4-D). A wound response in the presence of BA and 2,4-D at the time of culture was necessary for inducing shoot buds. The shoot bud regeneration was significantly influenced by size, type and orientation of explants on the culture medium. These shoot buds developed into 4–5 cm shoots upon transfer to a medium containing 1.1 μ*M* BA and 0.5 μ*M* α-naphthaleneacetic acid (NAA). The regenerated shoots formed rooted plantlets on MS medium supplemented with 4.9 μ*M* indole-3-butyric acid (IBA). About 15 plants were established in the field for further evaluation.

Key words: adventitious shoot regeneration; *Heracleum candicans*; petiole explants; xanthotoxin.

Introduction

Heracleum candicans Wall. (Apiaceae) is a perennial medicinal herb endemic to the northwest Himalayas. Its roots yield xanthotoxin which 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). The species propagates by seed, but seed germination is poor. This poor germination together with over-zealous collecting of plants from natural populations for commercial utilization threatens the existence of this plant species (Kaul et al., 1982). Very recently, micropropagation via axillary shoot proliferation (Wakhlu and Sharma, 1998) and somatic embryogenesis (Wakhlu and Sharma, 1999) have been reported. Here, we report the regeneration of *H. candicans* plants through adventitious shoot formation from petiole explants.

Materials and Methods

Shoot tips (1 cm long) from one adult plant of *Heracleum candicans* growing wild in Budhal (4300 m altitude), Jammu, India were surface-sterilized in 70% ethanol for 30 s and in 0.1% $HgCl_2$ for 2 min and washed five times in sterile distilled water. They were cultured on medium consisting of both salts and organics at the concentrations recommended by Murashige and Skoog (1962; MS), supplemented with 2.22 μ*M* 6-benzylaminopurine (BA) and 0.5 μ*M* α-naphthaleneacetic acid (NAA) for the induction of axillary shoots. Petiole and leaf explants were cut from the *in vitro*-formed axillary shoots and used as explants for shoot bud formation.

For induction of shoots, the explants were cultured on MS medium supplemented with BA or kinetin (2.22–9.29 μ*M*) either individually or in conjunction with 2,4-dichlorophenoxyacetic acid (2,4-D; 0.45–2.26 μ*M*), NAA (0.54–2.69 μ*M*), indole-3-butyric acid (IBA; 0.49–2.46 μ*M*) or indole-3-acetic acid (IAA) (0.57–2.87 μ*M*). The medium contained 3% (w/v) sucrose and the pH was adjusted to 5.8 before solidifying with 0.8% agar (Bacteriological grade, Ranbaxy Laboratories Ltd., S.A.S. Nagar, India). Molten medium (40 ml) was poured into 100-ml Erlenmeyer conical flasks and autoclaved at 103.4 kPa at 121°C for 15 min. The cultures were incubated at a temperature of 25 ± 1°C and 50–55% relative humidity under 16-h photoperiod of 30 μmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Bajaj Electricals Ltd., Mumbai, India).

Different explant types (petiole and leaf) and different explant lengths (2, 4, 10, 15, and 20 mm) were tested for shoot formation. The explants were cultured either intact or as transverse sections after cutting each explant into 2–20-mm explants. The effect of wounding on shoot regeneration was assessed by making four or five transverse cuts (partial) on the explants at 3–4 mm intervals.

To test the optimum age of shoots for shoot induction from excised petioles, explants were taken at weekly intervals after *in vitro* culture on medium containing 2.22 μ*M* BA and 0.5 μ*M* NAA. The influence of explant orientation was investigated by placing explants in horizontal, polar (basal side of explant down in the medium) and apolar (basal side of the explant up) positions on a medium fortified with 4.4 μ*M* BA and 1.1 μ*M* 2,4-D. Shoot buds were excised from explants and transferred to MS medium with 1.1 μ*M* BA and 0.5 μ*M* NAA for further growth and development. For rooting, 3–5-cm long shoots were transferred to MS medium supplemented with 4.9 μ*M* IBA.

Plantlets were planted in plastic pots (6 cm diameter) containing either vermiculite, sand, garden soil, vermiculite–garden soil (1:1) or vermiculite–sand (1:1). Plants were covered with perforated polyethylene bags for 1 wk and watered every 2 d. Hardened plants were established in pots containing sand, garden soil and farmyard manure (1:1:1). Data were taken as percentage of surviving plants after 4 wk.

All experiments were of completely randomized design and repeated at least twice. Each treatment consisted of 10 explants (two explants per 100 ml flask). The percentage of explants forming shoot buds and the mean number of shoot buds per explant was recorded after 4 wk of culture. Percentage data were subjected to arcsin transformation for proportions before analysis by ANOVA and then converted back to percentages for presentation in the tables (Snedecor and Cochran, 1968). Treatment means were statistically compared using Duncan's new multiple range test (Duncan, 1955).

Results and Discussion

Petiole explants excised from leaves of *in vitro*-grown plants enlarged in size and turned dark green within 2 wk of culturing. Shoot buds emerged directly from the surface of explants as small

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TABLE 1

EFFECT OF GROWTH REGULATORS ON ADVENTITIOUS SHOOT BUD FORMATION FROM NON-WOUNDED PETIOLE EXPLANTS (10 mm LONG) OF *HERACLEUM CANDICANS* AFTER 4 WK OF CULTURE

Growth regulator, BA (μM)	Explants producing shoot buds (%) (auxin, μM)[z]				Number of shoot buds per regenerating explant (mean±SE)			
	2,4-D	NAA	IBA	IAA	2,4-D	NAA	IBA	IAA
0.00	0 a (0)	0 a (0)	0 a (0)	0 a (0)	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
2.22	0 a (0)	0 a (0)	0 a (0)	0 a (0)	0 ± 0 a	0 ± 0 a	0 ± 0 a	0 ± 0 a
4.40	10 b (0)	10 b (0)	10 b (0)	10 b (0)	2.2 ± 0.5 c	2.2 ± 0.5 b	2.2 ± 0.5 b	2.2 ± 0.5 c
8.80	5 ab (0)	5 ab (0)	5 ab (0)	5 ab (0)	1.1 ± 0.4 b	1.1 ± 0.4 ab	1.1 ± 0.4 ab	1.1 ± 0.4 ab
2.22	20 c (0.45)	17 c (0.54)	21 c (0.49)	0 a (0.57)	4.3 ± 0.3 d	2.7 ± 0.2 b	3.7 ± 0.2 c	0 ± 0 a
4.40	80 f (0.45)	67 e (0.54)	76 h (0.49)	3 a (0.57)	14.6 ± 0.5 f	9.8 ± 0.4 e	10.0 ± 0.5 f	0.5 ± 0.4 ab
8.80	60 e (0.45)	49 d (0.54)	68 f (0.49)	10 b (0.57)	8.3 ± 0.4 e	6.8 ± 0.3 c	6.3 ± 0.3 d	1.2 ± 0.3 b
2.22	40 d (1.13)	72 f (1.34)	43 d (1.23)	14 bc (1.42)	9.1 ± 0.2 e	13.7 ± 0.4 g	7.9 ± 0.3 e	2.3 ± 0.3 c
4.40	90 g (1.13)	75 fg (1.34)	43 d (1.23)	18 c (1.42)	20.1 ± 0.6 h	17.6 ± 0.4 h	17.7 ± 0.4 i	4.2 ± 0.2 d
8.80	90 g (1.13)	78 fg (1.34)	71 fg (1.23)	18 c (1.42)	16.9 ± 0.7 g	12.6 ± 0.5 f	9.5 ± 0.4 ef	3.6 ± 0.2 d
2.22	80 f (2.26)	52 d (2.69)	70 fg (2.46)	23 de (2.85)	13.7 ± 0.5 f	8.0 ± 0.3 d	12.1 ± 0.4 g	4.9 ± 0.3 e
4.40	85 fg (2.26)	67 e (2.69)	74 gh (2.46)	29 f (2.85)	18.1 ± 0.6 g	14.4 ± 0.3 g	14.3 ± 0.4 h	8.5 ± 0.3 g
8.80	60 e (2.26)	67 e (2.69)	55 e (2.46)	27 ef (2.85)	9.0 ± 0.4 e	8.8 ± 0.2 de	8.7 ± 0.6 e	7.0 ± 0.1 f

[z] Auxin concentrations used are given in parentheses.
Means with the same *letter* within a *column* are not significantly different from each other at the 5% level by ANOVA and Duncan's new multiple range test.

green protuberances within 1 wk. The type and concentration of cytokinins added to the medium had a significant effect on shoot induction. BA was more effective than kinetin. In the absence of auxin, the highest percentage of explants forming shoot buds (10%) and the highest number of shoot buds per explant (2.2) were obtained with 4.4 μM BA. Among the four auxins tested for shoot induction, 2,4-D was the most suitable (Table 1). The maximum percentage of explants forming shoot buds (90%) and maximum number of shoot buds per explant (20.1) were obtained with 1.1 μM 2,4-D in combination with 4.4 μM BA. A similar promotive effect of the combined presence of an auxin and a cytokinin on adventitious shoot formation has been noticed in *Paulownia* spp. (Dimps Rao et al., 1996), *Vigna radiata* (Mathews, 1987) and *Asclepias syriaca* (Tepper and Knapp, 1992). Shoot bud formation did not occur in the absence of plant growth regulators as has also been earlier demonstrated in *Acampe praemorsa* (Nayak et al., 1997). As an explant type, petiole was the best explant in terms of percentage of regenerating explants (73%) and the number of shoot buds per explant (21.2) (Table 2). Of the different explant sizes assessed, 10 mm long petiole explants evoked the best response. The effect of wounding was tested on petiole (20 mm long) and leaf (20 mm × 20 mm) explants and was found to be significant for triggering differentiation of shoot buds (Table 3). Wounding appeared to establish regeneration sites in the explants. Similar results have been reported for *Garcinia mangostana* (Goh et al., 1994). The age of the explants was

TABLE 2

EFFECT OF EXPLANT TYPE AND SIZE (NON-WOUNDED) ON ADVENTITIOUS SHOOT BUD FORMATION IN *HERACLEUM CANDICANS* AFTER 4 WK OF CULTURE

Explant type	Explant size[z] (mm)	Explants producing shoot buds (%)	Number of shoot buds per regenerating explant (mean±SE)
Petiole	2	19 c	4.1 ± 0.3 c
	4	30 d	7.7 ± 0.3 d
	10	73 e	21.2 ± 0.3 g
	15	36 d	10.7 ± 0.7 e
	20	8 b	1.2 ± 0.5 ab
Leaf	2×10	0 a	0 ± 0 a
	4×10	5 b	1.0 ± 0.5 ab
	10×10	33 d	15.9 ± 0.6 f
	15×10	18 c	6.7 ± 0.3 d
	20×10	9 b	1.6 ± 0.5 b

[z] MS medium containing 4.4 μM BA and 1.13 μM 2,4-D was used.
Means with the same *letter* within a *column* are not significantly different from each other at the 5% level by ANOVA and Duncan's new multiple range test.

TABLE 3

EFFECT OF WOUNDING ON ADVENTITIOUS SHOOT BUD FORMATION FROM PETIOLE AND LEAF EXPLANTS OF *HERACLEUM CANDICANS* AFTER 4 WK OF CULTURE

Explant type[z]	Explants producing shoot buds (%)	Number of shoot buds per regenerating explant (mean±SE)
Petiole (20 mm long)		
Non-wounded	8 a	1.2 ± 0.5 a
Wounded	73 c	17.8 ± 0.4 c
Leaf (20 mm × 20 mm)		
Non-wounded	9 a	1.6 ± 0.5 a
Wounded	60 b	12.6 ± 0.4 b

[z] MS medium containing 4.4 μ*M* BA and 1.13 μ*M* 2,4-D was used.

Means with the same *letter* within a *column* are not significantly different from each other at the 5% level by ANOVA and Duncan's new multiple range test.

TABLE 4

EFFECT OF AGE OF DONOR SHOOTS ON ADVENTITIOUS SHOOT BUD FORMATION FROM NON-WOUNDED PETIOLE EXPLANTS (10 mm LONG) OF *HERACLEUM CANDICANS* AFTER 4 WK OF CULTURE

Age of donor shoot (wk)[zy]	Explants producing shoot buds (%)	Number of shoot buds per regenerating explant (mean±SE)
2	49 a	17.8 ± 0.3 c
3	88 c	20.8 ± 0.3 d
4	84 c	21.0 ± 0.5 d
5	59 b	13.8 ± 0.6 b
6	43 a	8.7 ± 0.4 a

[z] MS medium containing 4.4 μ*M* BA and 1.13 μ*M* 2,4-D was used.

[y] Age of the donor shoot refers to the number of weeks of explanting.

Means with the same *letter* within a *column* are not significantly different from each other at the 5% level by ANOVA and Duncan's new multiple range test.

observed to significantly influence shoot induction. Petiole explants excised from shoots cultured *in vitro* for 3–4 wk were more responsive than those from shoots cultured for 5 wk (Table 4). The higher response from 3–4-wk-old shoots may be due to the fact that they are physiologically active and easily influenced by the presence of exogenous hormones (Dong and Jia, 1991). Explants placed horizontally on the medium gave better results (100%) than those placed in a polar orientation (40%) (Table 5). The explants placed in an apolar position failed to regenerate shoots. The effect of explant orientation in adventitious shoot formation is well documented in *Lachenalia* spp. (Niederwieser and Staden, 1990), *Vigna radiata* (Gulati and Jaiwal, 1990), *Cucumis melo* (Yadav et al., 1996) and *Tamarindes indica* (Jaiwal and Gulati, 1991).

The shoot buds were not able to elongate when the explants bearing them were maintained on the induction medium (4.4 μ*M* BA and 1.1 μ*M* 2,4-D) for more than 4 wk. These buds elongated only when the explants were transferred to MS medium containing 1.1 μ*M* BA and 0.5 μ*M* NAA after 4 wk of culture. A maximum of 20 shoots were recovered from each explant on this medium. The

TABLE 5

EFFECT OF ORIENTATION OF PETIOLE EXPLANTS (NON-WOUNDED) ON ADVENTITIOUS SHOOT BUD FORMATION OF *HERACLEUM CANDICANS* AFTER 4 WK OF CULTURE

Explant orientation[z] (10 mm long)	Explants producing shoot buds (%)	Number of shoot buds per regenerating explant (mean ± SE)
Horizontal	100 c	21.4 ± 1.2 c
Polar (basal side down)	40 b	6.5 ± 0.3 b
Apolar (basal side up)	00 a	00 ± 00 a

[z] MS medium containing 4.4 μ*M* BA and 1.13 μ*M* 2,4-D was used.

Means with the same *letter* within a *column* are not significantly different from each other at the 5% level by ANOVA and Duncan's new multiple range test.

TABLE 6

EFFECT OF POTTING MIXTURE ON SURVIVAL AND HEIGHT OF *IN VITRO*-RAISED PLANTLETS OF *HERACLEUM CANDICANS* AFTER 6 WK OF TRANSFER TO *EX SITU* CONDITIONS

Potting mixture	Number of plants transferred	Survival rate (%)	Height (mean cm±SE)
Sand	20	5 a	8 ± 0.0 b
Garden soil	20	15 b	10 ± 0.8 b
Vermiculite	20	60 d	12 ± 0.5 b
Vermiculite–sand (1:1)	20	00 a	00 ± 00 a
Vermiculite–garden soil (1:1)	20	25 c	11 ± 0.4 b

Means with the same *letter* within a *column* are not significantly different from each other at the 5% level by ANOVA and Duncan's new multiple range test.

prolonged culturing of shoot buds on medium containing high levels of BA has previously resulted in the formation of abnormal shoots in *Citrullus vulgaris* (Dong and Jia, 1991).

All of the regenerated shoots which were placed onto MS medium supplemented with 1.4 μ*M* IBA formed roots within 4 wk. No roots were formed on shoots on a medium devoid of growth regulators. Roots appeared within 2 wk of culture on shoots incubated in the dark. Sixty percent of the plantlets (12 out of 20) were successfully hardened in vermiculite (Table 6). Sand plus vermiculite was ineffective for survival of regenerated plants. The hardened plants were established in pots (100%) containing a mixture of sand, garden soil and farmyard manure (1:1:1) and were 42 cm tall 6 mo. after transplantation. The results presented here are based on explants taken from one plant and the genotypic-dependent differences in response to culture have not been examined. The study, however, may be useful for developing a whole-plant regeneration system for the elite plants of this species.

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