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In vitro Multiplication of *Haloxylon recurvum* (Moq.) - a Plant for Saline Soil Reclamation

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Abstract

***Haloxylon recurvum* (Locally known as Khar) is drought and salt tolerant plant of Thar Desert. This plant is a major biomass producer and has economic and ecological importance for the region. There is need for study on biology, propagation and genetic improvement for utilization of this plant for reclamation of saline soils. We report here on *in vitro* propagation of *Haloxylon recurvum* (Moq.) using nodal explant. Secretion of phenolic compound from explants was a major constraint for establishment of culture. This was checked by thorough washing and quick transfer of explant on fresh culture medium. Juvenile nodal explant with leaves was found suitable for culture establishment. Benzyladenine(4.0 μM) incorporated in Murashige and Skoog (MS) medium with additives (50 mg/L ascorbic acid and 25 mg/L each of adenine sulphate, arginine and citric acid) induced multiple shoots from nodal explant. Addition of 1.0 μM naphthalene acetic acid (NAA) in combination with 4.0 μM BAP improved the growth of axillary shoots. Further shoot amplification was achieved by repeated subculture of mother explants on fresh medium. Forty percent of the micropropagated shoots rooted on half-strength MS medium with 4.0 μM indolebutyric acid (IBA) and 100 mg/L activated charcoal, at 28±2℃ and 60% RH. Sixty percent of these plantlets were hardened in green house.**

Key words: *Haloxylon recurvum*, halophyte, Sajji-khar, subculture, Thar Desert

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Introduction

Saline soils are the major problems of cultivated lands in semi-arid and arid areas. Thus there is a need to develop plant based solutions for reclamation of salt-affected lands. The native halophytes adapted to such conditions have, acquired morphological and physiological traits to thrive under salt-stressed conditions. Study of these plants, will help us understand mechanisms that enable them to tolerate adverse conditions. Such plants are natural source of gene(s) which can be utilized for prospecting. Breeding for tolerances to drought and salinity in crop- and forest-plants should be given high priority in biotechnology programs (Wang et al. 2003). The methods of somatic cell culture have been extensively used in plant breeding. The cell culture techniques provide alternative methods for screening and selecting cells/tissues/plants tolerant to drought and salinity. Cloning and large scale multiplication of selected germplasm is one of the important applications in plant biology. Cherian and Reddy (2003) studied salt tolerance of cell cultures of *Suaeda nudiflora*. Gu et al. (2004) investigated tolerance of suspended cells of *Populus euphratica* to ionic and osmotic NaCl-induced stresses. Stefaniak et al.(2003) initiated callus culture and induced organogenesis in two haloxerophytic *Asiatic Salsola*. Han et al.(2005) studied the effect of NaCl on cultured cells of *Suaeda salsa*.

Haloxylon recurvum Bunge ex. Boiss (Chenopodiaceae) is an important perennial plant of salt-affected areas of Indian desert. This is locally known as "Khar" (meaning salty; source of crude salts with sodium carbonate-Barilla or Sajji-Khar). The ash of the plant is used in laundry as a local substitute of soap. Aqueous solution of ash of this plant is used for treatment of ulcers (Bhandari 1990). The whole plant is preferred fodder for camel (Anonymous

1959; The Wealth of India). This plant is also a valuable source of fuel. *Haloxylon recurvum* is an efficient soil- binder and hence checks soil erosion. Above all, this plant provides valuable ecosystem services by facilitating habitat development for microbes under extreme xeric environment. This plant can be utilized for reclamation of saline soils/ waste lands.

Propagation of *Haloxylon recurvum* through seeds is poor, due to biotic and abiotic factors. Plants raised through seeds are slow in growth. There is need for development of conventional and non-conventional methods of propagation, genetic improvement and sustainable utilization of *H. recurvum*. We report here on micropropagation of *Haloxylon recurvum*.

Materials and methods

Plant Materials

Shoots of *Haloxylon recurvum* were collected from the Pachpadra Salt region of Rajasthan, (India) during March-April. Mature and juvenile nodal shoots (2-3cm with 1-2 nodes) with leaves intact or without leaves (leaves carefully excised) were washed with autoclaved water. These were surface sterilized with an aqueous solution of 0.1% (w/v) mercuric chloride (HgCl2) for 4-5 minutes and rinsed 3-4 times in autoclaved water. The surface sterilized explants were inoculated on MS (Murashige and Skoog 1962) medium containing additives (50 mg/L ascorbic acid and 25 mg/L each of adenine sulphate, arginine and citric acid) and 2.0, 4.0, 8.0 and 22.0 μM of benzylaminopurine (BAP) alone or in combination with 0.5 or 1.0or 2.0 μM of α -naphthalene acetic acid (NAA). Cultures were initially kept in the dark for 2-3 days and then under 30-35 μmol $m^{-2}s^{-1}$ irradiance (12 hour photoperiod) provided by fluorescent tubes at 26±2℃. The mother explants established in culture were transferred 4-5 times after an interval of 4 weeks on fresh medium for harvest of crops of shoots.

Rooting of micropropagated shoots

In vitro produced shoots (4-6 cm) were excised and transferred individually on one-fourth, half and full-strengths of MS basic salts containing 100 mg/L activated charcoal, and various concentrations (0.4, 2.4, 4.0, 9.0, 24.6 μM) of indolebutyric acid (IBA). These were incubated in the dark for 2-3 days and then under 30-35 μmol $m^{-2}s^{-1}$ irradiance at 28±2℃ temperature and 60% RH.

Acclimatization of *in vitro* produced plantlets

Plantlets of *H. recurvum*, generated in culture were taken out from the culture vessels and washed with sterile water to remove adhered nutrient media. These were transferred on autoclaved soilrite moistened with one-fourth strength of MS basal salts in glass bottles (135×70 mm diameter). The culture bottles were maintained in a green house for acclimatization of plantlets. Initially these bottles were placed near pad section (RH, 70-80%, temperature 30-32℃) and then gradually shifted towards fan section (RH 40-50% and temperature 32℃).

For each treatment five replicates were taken and each experiment was repeated thrice. The observations were taken regularly and data were recorded after 4 weeks of cultures. The data were subjected to statistical analysis; ANOVA (Gomez and Gomez 1976) .The relationship between variables i.e. chemical concentration, subculture phase (X) and response (Y) are examined using regression and correlation (Snedecor and Cochran 1967).

Results

Shoot induction and multiplication

Nodal shoots with fleshy leaves as compared to shoots without (excised) leaves proved to be better explants for establishment of culture. The excision of leaves and hence several injuries of the explant, caused browning of explants that leached phenolic substances in the culture medium. Both of these proved detrimental to the cultures. Incubation of the cultures initially in the dark and quick transfer of primary explant on to fresh culture medium checked browning of explants and the culture medium. Bud break occurred after 7-10 days of inoculation. Juvenile shoot of H. recurvum were found to be more responsive than the old and mature explants. About 80% of the explants derived from juvenile shoots produced multiple shoots from each node of explant. Maximum bud break and multiple shoot production was recorded on a medium with 4.0 μM of BAP (Table 1).The response of BAP on shoot number and length are significant (F=7.71 and 256.30, respectively). From each node, 2-3 axillary shoots differentiated (Figure 1a). BAP levels, above 4.0 μM caused callus formation at the bases of the explant, which in turn inhibited the growth of the axillary shoots. Growth of the shoots improved on a culture medium containing 4.0 μM of BAP and 1.0 μM of NAA (Table 2). The mother explants, after excision and harvesting of induced axillary shoots were repeatedly cultured 4-5 times. Number of axillary shoots from each node in-

Table 1. Induction of multiple shoots from nodal explant of *H. recurvum* on MS medium containing BAP and additives.

Concentration of BAP (µM)	Number of shoots (Induced) per Explant (mean ± SD)	Shoot length in cm (mean ± SD)
0.8	1.4 ± 0.48	0.34 ± 0.10
2.0	1.6 ± 0.48	0.9 ± 0.22
4.0	2.6 ± 0.48	3.06 ± 0.67
8.0	2.2 ± 0.74	2.38 ± 0.63
22.0	3.2 ± 0.74	0.74 ± 0.23
Computed F:		
Replication	2.571 ns	0.850 ns
Treatment	7.714**	256.289**
CD	0.793	0.219
	Log Y=1.486+0.247LogX (R^2=0.846; P<0.05)	Y=0.236+0.52X-0.023X^2 (R^2=0.688; P<0.1)

ns; non-significant,**; highly significant.

Y=number/length of shoots X=conc. of BAP

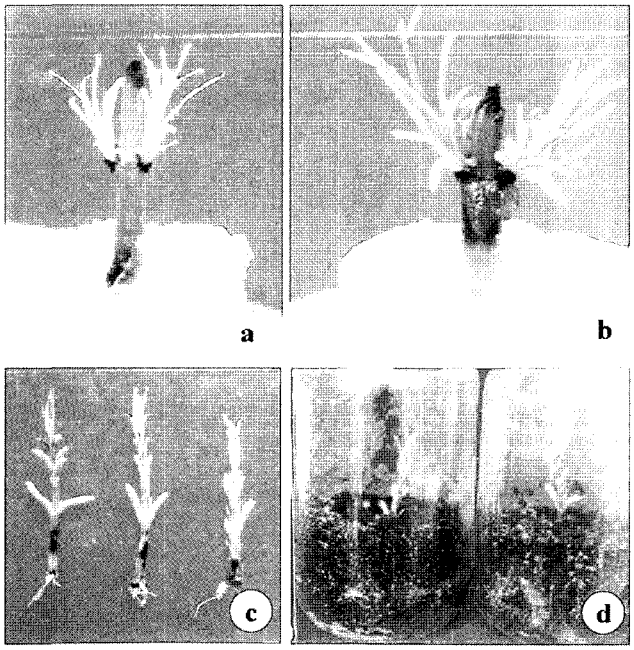

Figure 1. Micropropagation of Haloxylon recurvum. a. Shoot induction from nodal explant of *Haloxylon recurvum* on MS medium + 4.0 µM BAP + 1.0 µM NAA + additives. b. Multiple shoot differentiation by repeated transfer of mother explant on MS medium + 4.0 µM + 1.0 µM NAA + additives. c. Rooting of micropropagated shoots of *H .recurvum* on half-strength MS salts + 100 mgl^{-1} activated charcoal + 4.0 µM IBA. d. *In vitro* regenerated plantlets on soilrite moistened with one- fourth strength of MS salts, under hardening phase in green house.

Table 2. Effect of NAA concentration on growth of multiple shoots from nodal explant of *H. recurvum* on MS medium + 4.0 µM BAP + additives.

Concentration of NAA µM	Number of shoots per explant (mean ± SD)	Shoot length in cm (mean ± SD)
0.5	2.4 ± 0.48	2.4 ± 0.48
1.0	2.8 ± 0.4	5.38 ± 0.64
2.0	2.0 ± 0.63	3.6 ± 0.34
Computed F:		
Replication	0.586 ns	2.825 ns
Treatment	0.827 ns	129.285**
CD	-	

ns; non-significant,**; highly significant.

Table 3. Repeated transfer of mother explant and multiplication of shoots, of *H. recurvum* on MS medium containing 4.0 µM BAP + additives.

Transfer phase/stage	Shoot number per explant (mean ± SD)	Shoot length in cm (mean ± SD)
I	3.4 ± 0.48	2.62 ± 0.30
II	4.2 ± 0.74	2.3 ± 0.56
III	6.8 ± 0.74	3.6 ± 0.34
IV	6.2 ± 0.83	3.36 ± 0.38
V	5.0 ± 0.70	3.06 ± 0.32
Computed F:		
Replication	2.393 ns	6.962ns
Treatment	16.000**	23.919**
	1.047	0.324
	Y= -0.04+3.606X-0.514X^2 (R^2=0.821; P<0.05)	Y=1.656+0.837X+0.107X^2 (R^2=0.477; P<0.2)

ns; non-significant,**; highly significant.

Y=number/length of shoots X=transfer phase

creased on each successive subculture of primary explant. The repeated culture of mother explant contributed significantly for both shoot number (F=16) and shoot length (F=23.92). Three to four,4-5 and 6-8 shoots differentiated from each node of mother explant on first (Figure1b), second and third subcultures respectively (Table 3). After fourth and fifth subcultures, number of axillary shoots produced was low.

In vitro rooting and hardening of plantlets

Maximum of the micropropagated shoots rooted on Half-strength MS medium containing 4.0 µM IBA (Table 4 and Figure 1c).Concentration of IBA at 4.0 µM is critically significant for induction of roots. Incubation under the dark for initial 2-3 days at 28℃ promoted root induction from the shoots. Forty percent of the shoots rooted within 3 weeks. IBA concentration above 9.0 µM induced callus at the bases of the shoots. Sixty percent of the *in vitro* rooted plantlets were hardened in the greenhouse (Figure1d).

Discussion

Establishment of culture of *H. recurvum* is influenced by a number of factors. Physiological state of source plant and its age; season of collection of explant and its treatment are critical as these determine the growth and behavior of explant *in vitro*. It was difficult to surface sterilize the matured shoots as these have split bark that harbors recalcitrant microbes. The cut end of leaves and shoots exhibited excessive leaching of phenolic substances, a cause of browning of the culture medium-detrimental for cultures *in vitro*. In the present study, the browning was checked by culturing explant with leaves intact. Release of phenolic compounds from the cut end of explants were reduced by (i)thorough washing with autoclaved water (ii) by keeping the culture initially in the dark and (ii) quick transfer of explant to fresh culture medium. Keeping the cultures initially in the dark may help to reduce the browning problem (Adams et al.1979) by preventing or reducing the activities of the enzymes of both biosynthesis and oxidation of phenols (Krikorian1994). Maximum browning of explant and the medium occurred during initial two or three days of inoculation. Quick transfer of explants on fresh medium minimized browning. Rathore et al. (1991, 1992), Shekhawat et al. (1993) and Deora and Shekhawat (1995) applied this method for establishment of cultures of plants of arid regions. It is suggested that during aging and phase change, and also while surviving under adverse environmental conditions of winters/summers/stresses; the tissue(s) of these plants accumulate metabolites that inhibit bud breaking. These are released in culture and caused browning of media. These metabolic adaptations are also responsible for low responses *in vitro*. A suitable combination of auxin and cytokinins are important for organogenesis from axillary shoot explants (Park et al.2004). NAA is routinely used auxin for meristem and bud cultures (Hu and Wang 1983). A combination of BAP and NAA proved to be suitable for culture of node explants of *Haloxylon recurvum* for clonal shoot production. Repeated subculture of mother explants on fresh culture media is a potential method for multiplication of shoot in case of plants that are difficult to culture. Axillary shoots formation increased at each repeated transfer of mother explant, up to third-fourth subcultures. It is suggested that conditioning of the explant and dilution of inhibitors occur by repeated transfer. These activated the meristem and promoted shoot multiplication. Repeated subculture of explant has been suggested as one of the methods of rejuvenation of adult tissues (Deora and

Table 4. *In vitro* root induction from micropropagated shoots of *H. recurvum* on half-strength MS medium + 100 mg/L activated charcoal as affected by IBA concentration.

Conc. of IBA µM	Shoot response	Number of root per shoot (mean ± SD)	Root length in cm (mean± SD)
0.4	-	-	-
2.4	20	1.4 ± 0.48	0.74 ± 0.30
4.0	40	3.0 ± 0.63	0.74 ± 0.25
9.0	30	2.4 ± 0.48	0.34 ± 0.18
24.6	-	-	-
Computed F:			
Replication		1.048 ns	0.673ns
Treatment		18.135*	339.032**
CD		0.962	6.054
		$Y=0.359+0.472X-0.020X^2$ ($R^2=0.757$; $P<0.1$)	$Y=0.319+0.054X-0.003X^2$ ($R^2=0.396$; $P<0.3$)

ns; non-significant,**; highly significant.

Y=number/length of roots X=conc. of IBA

Shekhawat 1995).However, after 3rd subculture the shoot production from the mother explant declined.This was accompanied with callus formation and hyper-hydration of the explant.

Rooting and hardening of shoots is critical for micropropagation. The salts of culture medium,physical and chemical environment play important role in induction and elongation of roots. It has been found that half-strength of MS salts promote rooting of shoots (Gray and Benton 1991; Choi et al.1992). The cloned shoots of *Haloxylon recurvum* rooted on half-strength MS salts containing 100 mg/L activated charcoal and 4.0 µM IBA.Incubation in the dark promoted root induction from the shoots of *H. recurvum*. Dark treatment, particularly during the initial 3-7 days of root induction was reported to improve the rooting responses in several apple scion cultivars (Zimmerman1984; Zimmerman and Fordham1985). The Activated charcoal adsorbs substances presumed to be deleterious. Activated charcoal can provide a darkened environment for the growth of roots (Krikorian 1994).

The studies described in this paper are important for further research and development for germplasm conservation, large scale production of selected clones of *Horecurvum*. The *in vitro* methods of somatic cell culture may also be useful as tool for studying biology and mechanism of salt tolerance in this plant.

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