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Research Article

In-vitro Propagation of Himalyan *Hippophae salicifolia* Through Nodal Segments

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Abstract

Hippophae spp. (Seabuck thorn) is a deciduous, dioecious shrub that belongs to the family Elaeagnaceae, distributed in the temperate zone of Asia and Europe. The Indian Himalayas is believed to possess about 1,00,000 ha, the world's second largest seabuckthorn resources. Natural regeneration of *H. salicifolia* via seeds is scanty due to poor germination. The present investigation describes the successful in vitro propagation of *H. salicifolia* through nodal segments. Mercuric chloride ($HgCl_2$) solution (0.10% w/v) for 7 minutes treatment was proven to be the best for getting 80% uninfected green explants. MS (gelled with 0.7% Agar) + BAP (1.0 mg L^{-1}) + NAA (0.5 mg L-1) + Adenine Sulphate (50.0 mg L^{-1}) proved to be the best multiplication medium to produce 7.0 ± 0.45shoots per explant with average shoot length of2.38 ± 0.07 cm. For *in vitro* rooting ½ MS (gelled with 0.7% Agar) + IBA (1.0 mg L^{-1}) was proven the best medium for inducing maximum 60 ± 1.30% rooting with average 3.60 ± 0.09 roots per shoot. Plantlets were transferred to the field after *in vitro* hardening and acclimatization in the mist chamber and shade house.

Keywords: *Hippophae salicifolia*; *In Vitro* Propagation; Tissue Culture; Hardening; Acclimatization

Introduction

Hippophae spp., (family Elaeagnaceae) is distributed in the temperate zone of Europe and Asia particularly in the Himalayas of Jammu and Kashmir, Himachal Pradesh, Uttarakhand, and Sikkim at an altitude ranging between 2,100–3,600 m amsl [1-4]. The three main species of sea buckthorn distributed in India are *Hippophae rhamnoides, Hippophae salicifolia,* and *Hippophae tibetana*. Out of these, *H. salicifolia* is found in the Uttarakhand [5] under natural conditions confined to the river beds of Sukhi, Harsil, Gangotri, Mandakini Ghati, Alaknanda Ghati, Jamuna Ghati, Badrinath, Har-ki-Dun, Kalli Valley, Gori Valley, Buddhi, Byans, Darma, etc [6,7]. The flowering starts in the month of March, and fruiting starts from April, while ripening takes place during late October to early November that remains till March to April. The species bear red and yellow berries, which are edible, a rich source of vitamin C, carotenoids, minerals, vitamin B, vitamin E, and vitamin K and also used in the preparations of various products including local beverages [3]. Seeds contain high-quality oil which has many bioactive substances [8]. The flavonoids, fatty acids and other bioactive compounds of *H. salicifolia* berries might be capable of reducing the incidence of cancer [9]. The species is also considered as fine vegetation in improving the soil fertility and restoring degraded sites in high hills [10,11]. The natural regeneration of *H. salicifolia* via seeds is scanty and therefore the seed germination collected from a single population has been earlier studied [12]. However, to achieve higher productivity and select suitable genotypes for future breeding programmes, seed source testing is important [13]. The species is generally propagated by root suckers. However, extensive collection and a short growing season can hamper mass multiplication of the species. The availability of information on the similar aspects of *H. salicifolia* is scanty in the literature. Therefore, the present investigation was conducted on *in vitro* propagation of *H. salicifolia* through nodal segments.

Material and Methods

Description of the study area

Twigs of *H. salicifolia* were collected from the Garhwal region of Uttarakhand, viz., Hanuman Chatti (Chamoli); Dharasu and Yamunotri, both belong to the Uttarkashi district. Standard packing was practiced to transport the material to the Forest Research Institute

(FRI), Dehradun. Some twigs of *H. salicifoli*a were also collected from FRI campus. *In vitro* propagation study was conducted at the Tissue Culture Laboratory of Genetics and Tree Improvement Division, FRI, Dehradun. The area falls under a humid subtropical climate with an annual rainfall of 2073 mm.

Explant and its sterilization

Nodal explants were excised from twigs of *H. salicifolia* and washed under running tap water to remove the dust particles adhering to the surface. The explants were then washed with liquid detergent (Teepol, 5 –10 drops/100 ml.) followed by washing with Tween-20 (2 drops/100 ml solution) in gentle agitating conditions for about 5 minutes, and again washed under running tap water followed by fungicide treatment [0.1% Bavistin (50% carbendazim WP)] for 10 minutes. The explants were surface sterilized either with mercuric chloride solution or sodium hypochlorite (4% available chlorine) in various concentrations for different time duration. Finally, the explants were rinsed 4–5 times with sterilized distilled water to remove the traces of sterilant.

Axillary bud initiation and multiplication

After sterilization, explants were inoculated on MS medium supplemented with BAP (6-Benzylaminopurine) (0.5–1.5 mg L^{-1}) + NAA (α- Naphthalene Acetic Acid) (0.25–0.5 mgL-1) + Adenine Sulphate (Ads) (50.0 mgL^{-1}). *In vitro* grown shoots were multiplied through sub-culturing (transfer of cultures to a fresh medium) at periodic intervals of 3–4 weeks on MS (solid) medium supplemented with BAP (1.0 mgL^{-1}) + NAA (0.5 mgL^{-1}) + Ads (50.0 mg L^{-1}).

Culture medium and culture conditions

In all the experiments, MS (Murashige and Skoog) [14] medium supplemented with 3% sucrose as carbohydrate source and 0.7% bacteriological agar (HI Media) for solidification was used. Before addition of agar, the pH of the medium was adjusted to 5.8 using 1N NaOH or 1N HCl, which was autoclaved at 121^0 C with pressure of 15psi for 30 min. All the cultures were incubated for the photoperiod of 16 h in light (illuminated by 40-Watt cool white fluorescent tubes, 1200 lux) and for 8 h in dark under the culture room maintained at 23 ± 1^0C.

Rooting of *in vitro* grown shoots

In vitro regenerated shoots measuring 2–3 cm long were excised and kept for rooting on ½ MS medium supplemented with different concentrations (0.5–1.5 mg L^{-1}) of IBA, NAA and IAA.

Hardening and acclimatization

Plantlets need to be hardened and acclimatized before field transplanting, as they have grown within closed vials under high humidity and due to undeveloped cuticle lose water rapidly through the process of transpiration. Several experiments have been laid out to harden and acclimatize the plantlets, as the plantlets developed through tissue culture could not stand with the outer environmental condition.

Results

Sterilization of explant

Of the various concentrations of mercuric chloride and sodium hypochlorite at different time duration for surface sterilization of explant, the best result wasrecorded in 0.10% HgCl2 as explants remained green with 80% survivability (Table 1).

Sterilant	Concentration (%)	Treatment duration(in minutes)	Per cent uninfected explants	Condition of explants
$HgCl_2$	0.05	5	0	-
		7	0	-
		9	20	Green
	0.10	5	0	-
		7	80	Green
		9	40	Green
	0.15	5	100	Dead
		7	100	Dead
		9	100	Dead
NaOCl	10.00	5	0	-
		7	0	-
		9	0	-
	20.00	5	0	-
		7	0	-
		9	10	Green
	30.00	5	20	Green
		7	20	Green
		9	30	Dead

Table 1: Effect of different sterilants on survival of nodal explants of *H. salicifolia.*

Axillary bud initiation and multiplication

The analysis of variance (ANOVA) showed significant differences ($p>0.05$) between various combinations of auxin concentrations on shoot multiplication of nodal explants in *H. salicifolia* (Table 2).

Sources of Variation	Parameter: Rooting Percentage	
	MSS (df)	MSS (df)
Between auxin concentrations in various combination	2267.86* (6)	7.19* (6)
F value	675.53	187.65
P value (0.05)	0.000	0.000

Table 2: One-way ANOVA for effect of various combinations of hormones and additive on shoot multiplication of nodal explantsin *H. salicifolia.*

The Duncan Multiple Range Test (DMRT) was used to identify the significant differences between treatments (combinations of auxin concentration).The results of rooting (%) are presented in Table 3. Total seven hormonal treatments (BAP 0.0–1.5 mg L^{-1}, NAA 0.0–0.5 mg L^{-1} and Ads 50.0 mg L^{-1}) were tried for shoot initiation and multiplication of nodal segments. Data recorded after 4 weeks reveal that the maximum (7.0 ± 0.45) shoots per culture with average shoot length of 2.38 ± 0.07 cm was obtained in treatment No. 6, which is different from other combinations, i.e. hormonal combination BAP (1.0 mg L^{-1}) + NAA (0.5 mg L^{-1}) + Ads (50.0 mg L^{-1}) (Table 2; Plate 1. A-D). Some of the hormonal combinations are at par with each other and *vice versa*.

Sl. No.	MS medium +Hormone + Ads (mg L^{-1})			Average number of shoots per explant (Average ± S. Em.)	Average length of shoots(cm)
	BAP	NAA	Ads	After 4 weeks	After 4 weeks
1.	0.0	0.0	50	0.0 ± 0.00^{e}	0.0 ± 0.00^{a}
2.	0.5	0.25	50	4.0 ± 0.32^{d}	1.20 ± 0.10^{b}
3.	1.0	0.25	50	5.0 ± 0.45^{bc}	1.54 ± 0.07^{c}
4.	1.5	0.25	50	4.4 ± 0.25^{cd}	1.34 ± 0.05^{b}
5.	0.5	0.5	50	5.6 ± 0.25^{b}	1.76 ± 0.05^{d}
6.	1.0	0.5	50	7.0 ± 0.45^{a}	2.38 ± 0.07^{e}
7.	1.5	0.5	50	5.4 ± 0.25^{b}	1.54 ± 0.06^{c}

Table 3: Effect of hormones and additive Ads on shoot multiplication of nodal explants of *H. salicifolia*

*Superscript in the table followed by the same letter (a, b, c, d & e) showing non-significant values according to Duncan Multiple Range Test (DMRT)

Rooting of *in vitro* grown shoots

The ANOVA showed significant differences (p>0.05) between various combinations of auxin concentrations on rooting for *in vitro* regenerated shoots in *H. salicifolia* (Table 4).

Sources of Variation	Parameter: Rooting Percentage	
	MSS (df)	MSS (df)
Between Auxin concentration in various combination	24.19* (6)	2.61* (6)
F value	49.804	123.90
P value (0.05)	0.000	0.000

Table 4: One-way ANOVA for effect of various combinations of Auxins on rooting of *in vitro* regenerated shoots of *H. salicifolia.*

The DMRT showed significant differences between the treatments. Of the 3 auxins (IBA 0.5–1.5 mg L^{-1}, IAA 0.5–1.5 mg $L^{-1,}$ NAA 0.5–1.5 mgL^{-1}) tried for rooting of *in vitro* grown shoots, the IBA (1.0 mg L^{-1}) gave maximum (60 ± 1.30%) rooting with an average of 3.60 ± 0.09 roots per shoot after 8 weeks (Table 5; Plate 1.E). Again, results showed that few of the hormonal combinations are at par with each other and *vice versa.*

Sl. No.	½ strength MS medium +PGR (mg L^{-1})			Rooting Per cent	Average number of roots per shoot
	IBA	IAA	NAA		After 8 weeks
1.	0.0	0.0	0.0	0 ± 0.00^{e}	0.0 ± 0.00^{d}
2.	0.5	0.0	0.0	30 ± 0.63^{b}	1.50 ± 0.08^{b}
3.	1.0	0.0	0.0	60 ± 1.30^{a}	3.60 ± 0.09^{a}
4.	1.5	0.0	0.0	20 ± 1.10^{c}	1.00 ± 0.09^{c}
5.	0.0	0.5	0.0	5 ± 0.45^{d}	1.00 ± 0.09^{c}
6.	0.0	1.0	0.0	20 ± 1.10^{c}	1.70 ± 0.12^{b}
7.	0.0	1.5	0.0	0 ± 0.00^{e}	0.0 ± 0.00^{d}
8.	0.0	0.0	0.5	0 ± 0.00^{e}	0.0 ± 0.00^{d}
9.	0.0	0.0	1.0	0 ± 0.00^{e}	0.0 ± 0.00^{d}
10.	0.0	0.0	1.5	0 ± 0.00^{e}	0.0 ± 0.00^{d}

Table 5: Effect of auxins and their concentrations on rooting of *in vitro* regenerated shoots of *H. salicifolia*

*Superscript in the table followed by the same letter (a, b, c, d & e) showing non-significant values according to Duncan Multiple Range Test (DMRT)

Hardening and acclimatization

A number of experiments were setup for *in vitro* hardening and subsequent acclimatization of plantlets to *ex vitro* environment; the best result was achieved by transferring plantlets to ¼ MS medium having 2% sucrose followed by transfer to hycotrays containing vermiculite in mist chamber at temperature 30 ± 20C, RH 80-90%) after 2-3weeks and watering with 1/5 MS medium (Macro salts only).

Discussion and Conclusion

The study showed that the *in vitro* generated material through cuttings of matured shrub were transferred to the potted plantlets after proper hardening. The present work was in concurrence with an earlier study, that reported 100% rooting in *H. salicifolia* shoots raised from cotyledonary explants, but the cultures were actually taken from juveniles [15]. Tissueculture of other species of sea buckthorn such as, *H. rhamnoides* have been reported using axillary buds; however, low proliferation rate, vitrification, browning of medium, poor rooting, and genotypic differences among cultivars remain the main constraints [16-21]. Preliminary research reported that *in vitro* cultures of elite lines of *H. rhamnoides* have been established, but these failed and eventually died following subculture [22]. Recently, direct somatic embryogenesis was induced from leaves, cotyledons and hypocotyls of *H. rhamnoides* [23]. Issues of high levels of variations in establishing *in vitro* cultures of different promising cultivars of Indian sea buckthorn (*H. rhamnoides* spp. *turkestannica*), have been described in earlier research [24,25]. *Hippophae salicifolia* have been propagated using cotyledonary explant [15].

During the hardening and acclimatization process, tissue culture raised plantlets developed new leaves with well-developed cuticle which are capable of making organic food through the process of photosynthesis. High mortality has been observed upon transfer of plantlets to *ex vitro* conditions as the cultured plants have non-functional stomata, weak root system and poorly developed cuticle. A similarobservation was reported earlier [26], while working with the biological hardening and genetic fidelity testing of micro-cloned progeny of *Chlorophytum borivilianum*. Moreover, our study results into developed plantlets with full functional new roots (Plate 1. F-I).

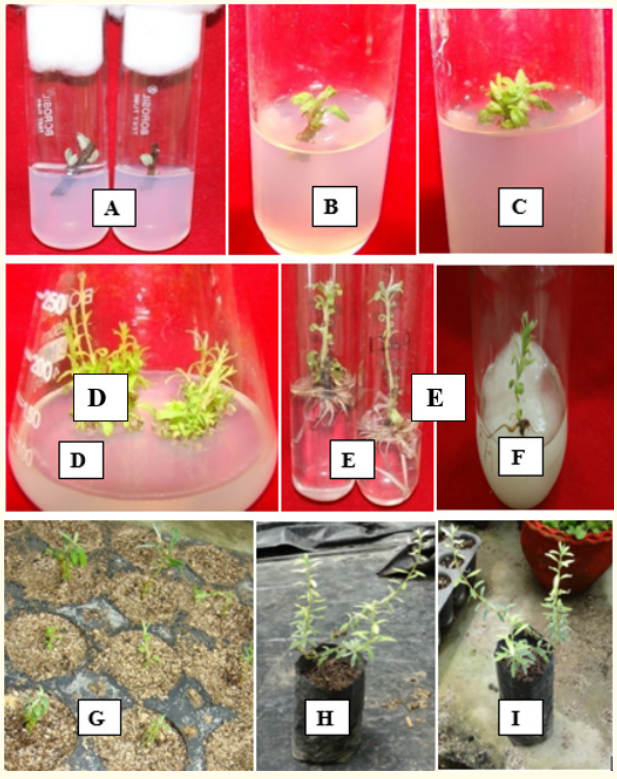

Plate 1: (A-I) In vitro propagation of *H. salicifolia* through axillary bud.

A. Bud induction from axillary node. B. Shoot initiation. C. Multiple-shoot formation. D. Multiplication of culture. E. *In vitro* rooting. F. *In vitro* hardening. G. Hardened plantlets. H & I. Pants ready for field transfer.

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