Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
https://www.phytojournal.com
JPP 2024; 13(1): 360-366
Received: 02-12-2023
Accepted: 04-01-2024

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A review on genetic diversity, micropropagation and transformations in the high-value medicinal plant of Himalayas-Seabuckthorn (*Hippophae* sp.)

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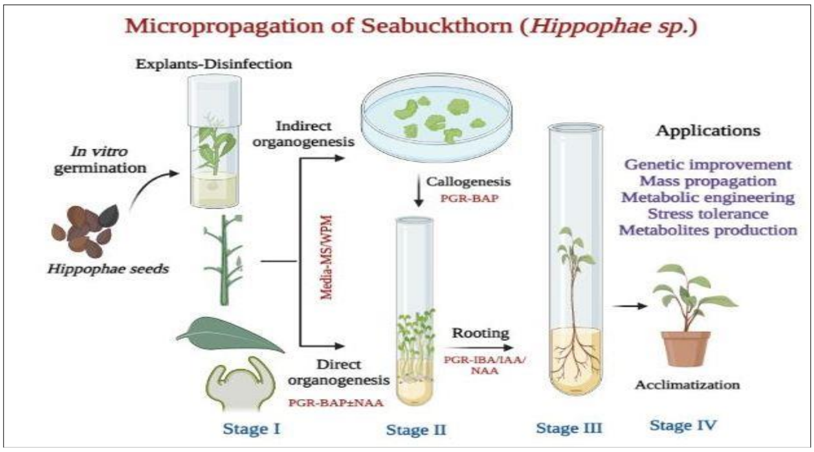
DOI: https://doi.org/10.22271/phyto.2024.v13.i1e.14849

Abstract
Seabuckthorn (*Hippophae* sp.) is popularly known as the "Gold Rush" for its commercial value. It holds well-established vegetative propagation and *in vitro* regeneration techniques. Accordingly, micropropagation of *Hippophae* sp. from multiple explants follows the developmental stages of establishment, shoot proliferation, and rooting. The successful *in vitro* propagation protocols utilized Murashige and Skoog medium and Woody Plant Medium supplemented with cytokinin for shoot organogenesis, somatic embryogenesis, and auxins for high rooting frequency. Subsequent acclimatization of *Hippophae* sp. with a reasonable survival rate was recorded under greenhouse conditions. The genetic transformations in seabuckthorn are yet to be studied in detail. Still, initial studies were performed with conventional methods using *Agrobacterium rhizogenes* (indirect) and biolistics (direct), which could support extensive transformation research in the future for commercialization and conservation. This short review provides an overview of compiled works on the tissue culture of *Hippophae rhamnoides* and *Hippophae salicifolia* that could assist in further assessment and vast applications.

Graphical Abstract



Keywords: *Hippophae rhamnoides, Hippophae salicifolia,* micropropagation, organogenesis, seabuckthorn

Introduction
Hippophae L. (Seabuckthorn) is a dioecious plant categorized under the Magnoliopsida class and the Elaeagnaceae family. The common name 'Seabuckthorn' has been designated from its thorny bearings and its distribution near seashores of European lands [1]. These species can adapt to temperatures ranging from +40°C to -40°C, drought, salinity, and alkalinity [2, 3]. Among all the species of seabuckthorn, *H. rhamnoides* is the most predominant and widespread, followed by *H. salicifolia* and *H. tibetana* in India and the major distributions are around the cold arid Leh-Ladakh of Trans Himalayan regions [4, 5]. They are usually spinescent, with rough bark, thick crown, and narrow alternate leaves with a silvery coating [6]. The extensive root system with reclamation ability also possesses nodules in symbiotic association with nitrogen-fixing *Frankia*, hence an actinorhizal plant [7].

Seabuckthorn species possess more than 190 bioactive compounds with nutraceutical and medicinal properties [8]. The colorful sour berries are rich in Vitamin A, E, and C, flavonoids, carotenoids, phytosterols, coumarin, catechins, leucoenthocyans, proanthocyanidins and fatty acids with high antioxidant properties [9, 10]. These phytochemicals are reported for a wide range of pharmacological activities such as anticancer, antibacterial, antiulcer, anti-inflammatory, antidiabetic, antiatherogenic, hypoglycemic, immune-modulatory, neuroprotective, cytoprotective, etc. China, Russia, and India are the largest producers and consumers of seabuckthorn as they process various products such as seed and fruit oil, raw juice, alcoholic beverages, candies, tea, jam, biscuits, vitamin C tablets, dry fruit, fruit wine, food colors, shampoos for commercialization [11]. The global demand for the 'multipurpose-wonder plant' increases its cultivation and propagation in India, China, Russia, North America, and several European countries [12].

Vegetative propagation in seabuckthorn

The traditional propagation of selective cultivars of seabuckthorn is achieved through seeds, softwood, hardwood cuttings, and root suckers (Shoots from roots- SFR), practiced majorly in cold regions [13]. The seed-germinated plantlets could not retain their valuable traits, and the reduction in seed viability increased with the storage time. Inspite of the significant loss of viability was controlled on storage for six years; seeds stored for 7-9 years have fertility loss [14]. The seed dormancy of seabuckthorn is a major limiting factor in vegetative propagation. Dale and Galic reported that pre-chilling of softwood cuttings for 6-8 weeks resulted in dormancy breaking with rooting up to 80% under greenhouse conditions [15]. The hardwood cuttings with less thickness (2.9 mm) showed a sevenfold increase with shooting and rooting in the absence of exogenous hormones [16]. Vegetative propagation from root cuttings and suckers is genetically identical (clones) to the parent plant, and these propagated plants may flower/fruit sooner than seed-propagated plants [13]. However, the adoption of conventional methods for propagation would be unfavorable during varied climatic conditions and the lack of natural pollinators throughout the year, leading to less successful cultivation. Though conventional vegetative propagation methods are simple, the success rates depends on the season; space required; availability of initial planting material, and cumbersome. Alternative methods like micropropagation or *in vitro* culture techniques are more effective and reliable in rapidly multiplying identical propagates [17].

Assessment of genetic fidelity

Seabuckthorn cultivation requires knowledge of gender and genetic traits for good yield. The analysis of seabuckthorn germplasm defines its quality traits, and the gender identification is significant as usually it could be revealed only during the flowering stage of 3-4 years. Microsatellite and molecular markers are used among all the *Hippophae* sp for the Polymerase Chain Reaction (PCR)-based detection [18]. RAPD markers were employed in *H. rhamnoides* ssp. *sinensis* [19, 20, 21], and ISSR markers for investigating three subspecies of *H. rhamnoides* [22]. Additionally, Korekar in 2012 developed two sex-linked SCAR markers for the early detection of its gender [14]. The gender and genetic fidelity of *H. salicifolia* was determined using SCAR and RAPD marker [23, 24, 25]. Though *H. tibetana* has not been studied much, its gender was identified with the SCAR markers, HrX1 and HrX2 [24]. Studies employing advanced SSR with EST markers detect high diversity in adaptive genes of seabuckthorn [26] than AFLP [27].

Micropropagation studies

In vitro culture technique has numerous advantages of developing large-scale identical clones, facilitating year-round production within minimum space and shorter duration over the vegetative methods. The purpose of micropropagation is to achieve disease-free and high yield of plants from cells and tissues (explants) of selected plants that are segregated, disinfected, and incubated in the specified growth-promoting medium under aseptic containments [28]. The success of micropropagation involves several factors, as the composition of the culture medium, culture environment, and genotype. The development of procedures for rapid *in vitro* clonal micro propagation in seabuckthorn may be a great commercial success to the industries, as large-scale production of desired compounds from seabuckthorn could be achieved from basic techniques. Regular optimized protocols for every seabuckthorn species must be imposed to attain all the advantages of availability, mass propagation of selective clones and conservation of cultivars. Micro propagation in seabuckthorn generally follows the defined stages of growth and differentiation such as 1) Initiation of aseptic cultures, 2) Shoot induction, 3) Root induction, and 4) Acclimatization [29, 30]. The procedures and protocols developed under each stage of *in vitro* regeneration for *H. rhamnoides* and *H. salicifolia* under optimized conditions are delineated below.

Stage I: Initiation of aseptic cultures

Selection of Explants

The selection of explants has been considered an essential factor in regeneration. All *Hippophae* sp. holds potential germination efficiency; hence seed and cotyledons, cotyledonary nodes (CN), and hypocotyls (HC) from the *in vitro* germinated seedlings are widely used. The direct explants such as leaves, meristem/shoot tips [31, 32], axillary buds [33], and nodal segments [34] were employed in investigating the shooting and rooting potential of each species.

Surface sterilization

All explants are subjected to chemical treatments during propagation to achieve contamination-free cultures. The chemical sterilant and the treatment duration vary based on the type of explants. All *Hippophae* sp. were commonly treated with tween 20, sodium hypochlorite (NaOCl), 70% ethanol (EtOH), and mercuric chloride ($HgCl_2$), while bavistin (0.1%) antibiotic (fungicide) was used in addition for *H. salicifolia* explants. The standard treatment methods followed to obtain contamination-free cultures of *Hippophae* sp. are listed in Table 1.

Table 1: Different treatment procedures followed for surface sterilization of explants

Treatments	Explants	References
H. rhamnoides		
40% Sulfuric acid (4 min)	Seeds	[35]
5% NaOH (10 min), 70% EtOH (1 min)	Meristem/Shoot tips	[31]
2 drops of Tween 20, 70% EtOH (5 min), 30% NaOCl (1hr)	Seeds	[36]
2% NaOCl+0.03% Tween 20 (30 min) and 70% EtOH (2 min)		[12, 37]
Tween 20 (20 min), 0.5% Bavastin (20 min), 1-2% NaOCl, 0.2% $HgCl_2$ (5 min) and 70% EtOH (3 min)		[38]
0.1% Teepol, Ascorbic acid+ Ctric acid (2 hrs) in agitation, 2.5 mg/mL Tetracyclin (2 hrs) at low temp, 70% EtOH (4 min), 0.1% $HgCl_2$ (6 min)	Axillary buds	[33]
H. salicifolia		
0.5% NaOCl (2 min)	Seeds	[39]
0.1% Tween 20+ 50% NaOCl (20 min), 1% Bavistin (1 hr), 70% EtOH (70 sec), 0.01% $HgCl_2$ (3min)		[24. 40]
70% EtOH, liquid detergent (5 min), 0.1% $HgCl_2$ (3-5 min)		[41]
Teepol (5 min), 0.1% Bavistin (10 min), 4% NaOCl/ $HgCl_2$	Nodal segments	[34]

Cultural medium and Growth conditions: The synthetic media with constituents of minerals, nutrients, and carbon sources, along with the plant growth regulators (PGRs), have been prepared for rapid and ultimate responses in propagation. *H. rhamnoides* has been optimized with differential media such as MS [42], Woody Plant medium (WPM), and plain media (PM) for differentiation, while WPM is highly preferred for *H. salicifolia*. Media supplements and incubation conditions influence growth rate and differentiation efficiency of different explants. Irrespective of any regenerative stages, the medium containing 3% sucrose and 0.7-0.8% gelling agent with a final pH of 5.5-5.8 has been generally used in all the studies. And the incubation conditions of temperature ranging from 20-26°C, under the photoperiod of 16/8 hr light /dark, are adopted. Further, the standardized *in vitro* conditions maintained for both *Hippophae* sp. are tabulated in Table 2.

Table 2: Culture medium and growth conditions optimized in various studies

	Medium	Growth conditions	Study	References
H. rhamnoides	MS	26/20°C, 16h photoperiod; photon flux density- 30µE/m^2/s	Germination and shoot induction	[35]
	1/4 MS		Rooting	
	SH	25±2°C;16h photoperiod; photon flux density- 55µmol/m^2/s	Somatic Embryogenesis; Shoot organogenesis	[36]
	PM	20°C, 16h photoperiod; photon flux density- 60-80 µmol/m^2/s	Pre-culturing	[12]
	MS		Shoot organogenesis	
			Rooting	
	1/4 MS+3% AC	25±2°C, 12h photoperiod; photon flux density- 40µmol/m^2/s	Seed Germination	[38]
	PM+100 ppm Inositol	18°C, 16/8h photoperiod	Pre-culturing	[33]
	WPM		Shoot induction	
	PM (agar+Gelrite)	20°C, 16h photoperiod; photon flux density- 100 µmol/m^2/s	Pre-culturing	[37]
	WPM		Shoot organogenesis	
H. salicifolia	WPM	24±2°C, 16/8 hr photoperiod; photon flux density- 30 µmol/m^2/s	Shoot induction and proliferation	[24])
	MS+50 mg/L AS	23±1°C, 16/8 hr photoperiod; intensity- 1200 lux	Multiple shoot induction	[34]
	PM- agar+0.01% Myoinositol	25±2°C, 16/8 hr photoperiod; 70- 80% relative humidity; photon flux density of 40 µmol/m^2/s	Pre-culturing	[40]
	MS/WPM		Shoot induction	
	½ MS		Root induction	
	MS+0.2% AC	25±2°C, 16/8 hr photoperiod; 60±5% humidity; intensity- 2500 lux	Direct organogenesis	[41]

AC-Activated charcoal, AS- Adenine sulphate, MS- Murashige & Skoog, PM- Plain medium, PVP- Polyvinylpyrrolidone, WPM- Woody plant medium

Control of media browning: Browning of culture medium is extensively observed during micro propagation of Seabuckthorn species, negatively impacting the development of *in vitro* cultures [42]. This major constraint was controlled by media fortifications and supplementation with activated charcoal (AC), storing seeds at 4°C, and using of SH medium [36]. Prior treatment of the explants with ascorbic acid and citric acid (1500 mg/L) for 1-2 hrs at low temperatures has a better effect on phenolic accumulation [40, 33]. *H. salicifolia* has controlled for phenolic release using polyvinyl pyrrolidone (PVP) at a concentration of 100 mg/L in a culture medium also aiding in growth enhancement [25]. Although specific treatments and media components are used, regular subculturing or change of media every 3-4 weeks squelches phenolic release and ensures a healthy state of cultures [41]. Excess browning can cause vitrification, a physiological defect occurring in the tissues. Also, factors such as gelling agents, hormones, organic and inorganic compounds, temperature, light, and water potential decide the cause of vitrification [44]. The decrease in explant vitrification of *H. rhamnoides* has been overcome by propagating the explants in ½MS through MS and finally to WPM medium [33].

Stage II-Shooting

Seed germination studies: Montpetit and Lalonde initially propagated *H. rhamnoides* seeds in the synthetic medium under *in vitro* to induce plantlets as a source of explants with no optimizing conditions [35]. Presoaking treatments were investigated to improve the seed germination in seabuckthorn. Better results were obtained with stratification (4°C) while soaking in (Gibberllic acid) GA_3 shortened the mean germination time. The highest germination was achieved in potassium nitrate (KNO_3) and Thiourea (100 mM), with 83.3% [45]. Breaking of seed dormancy was achieved by seed treatments with Thiourea (1%) and KNO_3 (0.1%), also enhancing the mean germination% [39]. Apart from chemical treatments, physical parameters were investigated on germination patterns of *H. salicifolia*. Temperature and various color light responses were noted in which the maximum sprouting was achieved under red light followed by yellow. But the length of radical and plumule was highest in control (white light) with an optimized temperature of 25°C [46, 47]. Germination of *H. rhamnoides* under *in vivo* (soil-rite) and *in vitro* (1/4 MS medium) showed 73% and 90%, respectively [38].

Shoot organogenesis: The initial regeneration stage is to establish microbe-free shoots from various explants in a suitable medium. The direct shoot induction takes about an average of 4-6 weeks and prepares the explants for stage II (multiplication), where shoot proliferation and multiple shoot clusters are induced from direct explants or callus upon subculturing [48]. *H. rhamnoides* has been studied for direct shooting in which the highest shooting frequency of 12 shoots/leaf explant was reported in MS medium with Benzyl adenine purine (BAP) and Thidiazuron (TDZ) each of 2.2 µM (~0.5 mg/L) in combination [12], whereas *H. salicifolia* has been reported the highest multiplication in BAP (1.1 mg/L) + α-Naphthalene acetic acid, NAA (0.5 mg/L) + Adenine Sulphate (100 mg/L) with an average of 22 shoots. The additional media component AS directly impacts multiple shootings [41]. Table 3 lists the overall *in vitro* experiments in *H. rhamnoides* and *H. salicifolia*. Similar results were observed for multiple shooting from BAP supplemented medium in berry bearing plant, *Elaeagnus angustifolia* [49, 50]. The species of Elaeagnaceae family or the oleaster are majorly propagated vegetatively (hardwood cutting), while only few were studied for their *in vitro* shootings. Hence application of BAP could be a suitable choice of PGR for shoot organogenesis in relative species.

Table 3: Summary of *in vitro* study of *Hippophae* sp. (Stage II & III of micropropagation)

Species	Explants	Media	Plant Growth Regulators (PGRs) mg/L								Study	Results	References
			BA	TDZ	Kn	NAA	IAA	IBA	GA_3	CPPU			
H. rhamnoides	*In vitro* Shoots	MS	0.22	-		-	-	-	-	-	Shoot Multiplication	No of shoots- 3-5/explant	[35]
		¼ MS+29.2mM Sucrose	-	-	-	18 µg/L	-	-	-	-	Rooting	No of roots- 30-50/shoot	
	Shoot tips	WPM	0.4-1.0	-	-	-	-	-	-	-	Multiplication and rooting	No of shoots- 3.3-4 and 33% rooting	[31]
	In vitro buds	MS/ ½ MS	0.3-0.5	-	-	0.05	-	-	-	-	Caulogenesis	-	[51]
			-	-	-	-	0.2	-	-	-	Rooting		
	Shoot tips	MS	0.1-0.25	-		-	-	-	-	-	Shoot induction and Multiplication	No of shoots- 3-4/explant	[32]
	CN, Leaf HC	SH	-	-	1	-	0.5	-	-	-	Direct somatic embryogenesis	All explants induced globular embryo without callus formation	[36]
	Young/ Adult Leaf	MS	0.5	0.22	-	0.1	-	-	-	-	Shoot organogenesis	No of shoots- 18/explant	[12]
			-	-	-	-	-	1.25	-	-	Rooting	No of roots- 5.1±0.8/shoot	
			2.9	-	-	0.1	-	-	-	0.5	Somatic embryogenesis	Globular embryo formed without callus formation	
	HC	WPM	1.1	-	-	-	-	-	-	-	Callus induction	-	[52]
			1.1	-	-	-	-	-	0.36	-	Caulogenesis	8 shoots/callus limp	
			-	-	-	0.1	-	-	-	-	Rooting	78% induced; No of roots- 2-3/shoot	
	Active buds	WPM	1	-	-	-	0.5	-	-	-	Multiple shooting	80% explants induced; No- of shoots-6.5/explant	[33]
			-	-	-	-	-	1.5	-	-	Rooting	66.7% induction	
	In vitro roots	WPM	1	-	-	-	5	-	0.07	-	Shoot organogenesis	-	[37]

Species	Explants	Media	Plant Growth Regulators (PGRs)								Study	Results	References
			BA	TDZ	Kn	NAA	IAA	IBA	GA_3	CPPU			
H. salicifolia	HC CN	WPM	0.1	-	0.5	-	5	-	0.1	-	Multiple shoot induction	No. of shoots- 21.6/explant Avg. length- 3.3 cm	[24]
		½ WPM	-	-	-	-	-	1	-	-	Rooting	No. of roots- 3.3/shoot	
	NS	MS + 50 mg/L AS	1	-	-	0.5	-	-	-	-	Multiplication	No of shoots- 70±0.45/explant Avg. length- 2.38±0.07 cm	[34]
		½ MS	-	-	-	-	-	1	-	-	Rooting	60% induced, No. of roots- 3.60±0.09/explant	
	Male & Female Bud	MS/WPM	10	-	-	-	3	-	2	-	Shooting	Male- 30%; Female 35%, No. of shoots- 4.60±0.24/explant	[40]
		½ MS	2	-	-	-	7	-	-	-	Rooting	No. of roots -3.20±0.27/shoot	
	CN	MS+0.2% AC	1.1	-	-	0.55	-	-	-	-	Direct organogenesis	No of shoots- 7.33/explant Avg. length- 5.23 cm	[41]
			-	-	-	-	-	0.6	-	-	Rooting	No of roots per shoot- 2.44/explant Length- 0.98 cm	
		MS+0.2% AC + 0.1% AS	1.1	-	-	0.55	-	-	-	-	Multiplication	No. of shoots- 22.56/explant Avg. length- 10.9 cm	
	HC		1.65	-	-	0.55	-	-	-	-	Caulogenesis	80% callus induced shoots	

MT- Meristem tips, NS- Nodal segments

Stage III-Rooting

Shoot proliferation is subsequently followed by rhizogenesis (Stage III) to improve the survival rate and acclimatization. Most reports showed that shoot propagates eventually rooted in shooting medium with minimal auxin concentration than cytokinin. The emergence of lateral/adventitious roots wer observed in almost every medium with the influence of IBA (Indole-3-butyric acid) and NAA individually or in combination. Optimized rhizogenesis of *H. rhamnoides* was noticed with IAA (Indole-3-acetic acid) and NAA at varied concentrations, while IAA and IBA were used for *H. salicifolia*. The specified concentrations for root induction from micro shoots are provided in Table 3. From the literature search the species *Elaeagnus angustifolia* has shown formation of lateral roots in IBA which can be the recommended root induction regulator for this family [49].

Stage IV: Acclimatization

Successful acclimatization is the ultimate goal of regenerated plantlets, which involves the transfer to the soil environment. Acclimatization refers to the stepwise or orderly transfer of plantlets to soil and adapts as autotrophic. The rooted propagates, after specified days of *in vitro* growth, are shifted to soil-rite [38], Sand: Vermicompost: Perlite (2:1:1) under 20°C [12], Sand: Perlite: Soil (1:1:1) under 30±2°C with 85% humidity [36], Soil: Farmyard Manure (3:1) [52] for 3-4 weeks. Even the nodulation of *Frankia* strains in rooted plantlets was studied by Montpetit and Lalonde after the hardening of seedlings to the soil [35]. Similarly, *H. salicifolia* seedlings were transferred to soil-rite followed by Sand: Vermicompost: Soil-rite (1:1:1) under greenhouse conditions [25], whereas Trivedi *et al*. (2020) [40] and Ajay *et al*. (2020) [41] suggested Sand: Soil: Manure (1:1:1). Chauhan *et al*. (2019) recommended to watering of hardened seedlings with 1/5 MS macro salts for 2-3 weeks to ensure healthy plants [34].

Preliminary genetic modifications

Gene introduction and stress-tolerant studies on seabuckthorn were initially performed with conventional methods that led to successful transformation. Stable trans formants of *H. rhamnoides* with insertion of lectin gene [53] and kanamycin resistance [54] are brought out by the standard transformation techniques (Table 4).

Table 4: Preliminary genetic transformations in *H. rhamnoides*

Gene of Insertion	Method of Transformation	Objectives	References
Pea lectin gene	*A. rhizogenes* 15834 mediated	Induction of hairy roots	[53]
Kanamycin resistance gene & β-galacturoniase	Particle bombardment (Gold nanoparticles)	Kanamycin resistant plants	[54]

Conclusion

Biotechnological advances have supported primary research and applications in seabuckthorn over decades. Including the taxonomical classification of its species, subspecies, and cultivars, identification through genetic markers, product development, vegetative propagations, etc, have been delineated throughout these years. As like other woody perennials, seabuckthorn cultivation gets affected by pathogens, climatic constraints, and other factors; hence the development of micropropagation techniques and genetically engineering methods is necessary to improve its stability and gene quality against biotic and abiotic factors [17]. This review has collated the successfully established protocols for regeneration of seabuckthorn species and the control of vitrification and browning. At times propagation methods alone do not meet the requirements of specified bioactive metabolites for industrialization, improving plant qualities, and understanding the specialized gene functions. The establishment of genetic improvements and transformation technologies through novel biotechnological tools, such as RNA interference (RNAi), trans-grafting, cisgenesis/ intragenesis, and genome editing, support the needs [55]. Transformations and molecular improvement in *Hippophae* sp. rely on promising *in vitro* regeneration protocols. Genetic transformations in seabuckthorn were confined to conventional methods, and novel genome editing tools were yet to be introduced in molecular methods [56]. Basic knowledge and understanding of *Hippophae* sp. under *in vitro* propagation provide the foundation for extensive studies for producing suspensions, haploids, and genetic improvements considering development or enhancement of specific metabolites and essential oils. On the other hand, being a highly tolerant species, propagation of seabuckthorn under extreme climate and vegetative conditions such as in lower altitude, high temperate and tropical lands requires insights that could be established in near future.

Abbreviations

RAPD-Random Amplified Polymorphic DNA, AFLP-Amplified Fragment Length Polymorphism, SCAR- Sequence Characterized Amplified Regions, SSR- Simple Sequence Repeats, EST- Expressed Sequence Tags, SH- Schenk & Hildebrant medium, IAA-Indole-3-acetic acid, GA_3-Gibberellic acid, TDZ- Thidiazuron, Kn- Kinetin, 2,4-D- 2,4-Dichlorophenoxyacetic acid, CPPU- N-(2-Chloro-4-pyridyl)-N1-phenyl urea.

Statement of declarations

Conflict of Interest

The authors declare that there are no competing interests.

Acknowledgement

We acknowledge to University Grants Commission -BSR, New Delhi, India for the support under Start-up grant - No.F-30-410/2018.

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