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Sea Buckthorn a Boon for Trans-Himalayan Region of Ladakh: A Review

Kunzang Lamo, Shivendu Pratap Singh Solanki

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ABSTRACT

Sea buckthorn (*Hippophae rhamnoides* L.) is an ecologically and economically important plant species and is currently being domesticated in various parts of the world including India. Sea buckthorn achieved a unique status in the trans-Himalayan Ladakh because of its immense medicinal and therapeutic potentials. High nutrient and medicinal values of the fruit attracts researchers to exploit in the field of biotechnology, neutraceutical, pharmaceutical, cosmetic and environmental sciences. However, unrestrained exploitation and even destruction of sea buckthorn resources leads to loss in its diversification and extinction. Therefore, there is need to conserve the wild genotypes of sea buckthorn to make them available as a source for breeding of new plant varieties in future and judicious utilization of wild species can bring more benefit to mankind throughout the world. Application of DNA markers to facilitate marker-aided selection (MAS) for diversity studies and identification of cold resistant genes have been developed, further improvements is still needed. Advances in plant genomics, genetic mapping and QTL analysis provide further means to improve the understanding of crop diversity at species and gene levels, it also accelerated the dissection of genetic control of important traits and development of high value genotypes in the context of breeding programs. Advances in biotechnology provided many tools that will benefit in providing quality planting material, generation of new cultivars and mass propagation therefore help in area expansion in areas like Ladakh. DRDO is an active organization in collaboration with other institutes to enhance the research field in sea buckthorn and grant patent on its products. There is no doubt that the future holds great promise for sea buckthorn cultivation in Ladakh and efforts need to be made to certify sea buckthorn as organic.

Key words: Advances, Biotechnology, Breeding, Importance, Ladakh, Sea buckthorn.

Sea buckthorn (*Hippophae rhamnoides* L.) is a dioceous shrub which belongs to family Elaegnaceae. The name is from its habit of growing near the sea and from the possession of many spines or thorns that are significant in some sea buckthorn species. In Ladakh, sea buckthorn is locally called as 'Tsermang' and the fruits are called as 'Tsestalullu'. The shrub serve as a ideal plant in Trans-Himalayan region of Ladakh, particularly for researchers in the field of biotechnology, neutraceutical, pharmaceutical, cosmetic and environment sciences (Stobdan *et al*., 2008). The region is characterized by extreme climatic conditions like high wind velocity, high rate of soil erosion, and extreme temperatures, i.e. –30°C in winter to +35°C in summer. Sea buckthorn can tolerate extreme temperature upto – 40°C to + 40°C. There are seven species of genus Hippophae, out of which three are found in India, namely *Hippophae rhamnoides*, *Hippophae salicifolia* and *Hippophae tibetana* (Dwivedi *et al*., 2009). Among these species *H. salicifolia* and *H. tibetana* are lesser explored species of sea buckthorn (Gupta *et al*., 2011). However, it was reported that *H. salicifolia* is rich in vitamin C content, and also contains all essential polyunsaturated fatty acids particularly omega-3 and omega-6, high quality berries with late maturing (Gupta *et al*., 2011). All species are diploid (2n = 24), cross pollinated (wind) and dioecious, which restricted to the Qinghai Plateau and adjacent areas, with the exception of the species *Hippophae rhamnoides* L. that occurs widely but sporadically in Asia and Europe (Stobdan, Korekar, and Srivastava, 2013). In world, the total acreage under sea buckthorn is reported about 3.0 million hectares (both wild and cultivated cover) and approximately 90% of world's sea buckthorn is found in China, Mongolia, Russia, Northern Europe and Canada. China being the leading producer among all the countries. In India, sea buckthorn grows naturally in different parts of Jammu & Kashmir, Himachal Pradesh, Uttrakhand, Sikkim and Arunachal Pradesh. However, its importance is came into limelight only after the Defence Institute of High Altitude Research (DIHAR), has transferred sea buckthorn-based technology to a private firm in year 2001, where berries were collected on large scale to generate more income. Since then, Ladakh remain the leading place with an estimated area of 13,000 ha (70 %) under sea buckthorn in the country. Although, the mean annual berry harvest is approximately 231 MT which is less than 5% of the total available sea buckthorn in the region. This is largely due to short harvesting season (20-30 days), which coincides with the harvesting time of other crops, due to lack of its appropriate plantation along with other causes like small berry size which makes harvesting a tedious process and

Division of Fruit Science, Punjab Agricultural University, Ludhiana-141 004, Punjab, India.

Corresponding Author: Kunzang Lamo, Division of Fruit Science, Punjab Agricultural University, Ludhiana-141 004, Punjab, India. Email: Kunzanglamo1490@gmail.com

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labour intensive, which mainly restricts the total harvest of the plant. The berry is very delicate and highly perishable in nature therefore, long distance transportation of fresh berry is challenging. Many processed products like Leh berry beverage, herbal tea, jam etc. were successfully prepared and grant patent on these products. To prevent further post-harvest losses there is a need of cold chain facilities to extend its availability and development of value added products. At present, there is high demand for sea buckthorn which exceeds the supply. To meet the demands for sea buckthorn, studies have been undertaken at DIHAR to meet the long term goal of mass cultivation of sea buckthorn in Ladakh. Different projects are still undertaking to convert the vast barren land into green patch by planting sea buckthorn in areas including Nubra, Leh, Changthang and Suru. Since sea buckthorn is a relative new-comer as a crop, a little is known about its cultivation practices. Furthermore, there is no available high yielding varieties that can be cultivated and and propagated on a large scale.

Nutritional values

Sea buckthorn is mentioned in the writings of ancient Greek scholars such as Theophrastus and Dioscorides. Sea buckthorn is very well known for its medicinal values since early 8 century in the Tibetan medicinal classic *rGyud Bzi.* In Ladakh region, even today *Amchies* (local traditional doctors) often prescribed preparations from sea buckthorn for treatment of many diseases. Sea buckthorn has been reported to contain more than 190 bioactive compounds in the seeds, pulp, fruit, and juice (Gupta *et al.,* 2005). These compounds include fat-soluble vitamins (A, K, E), 22 fatty acids, 42 lipids, organic acids, amino acids, carbohydrates, Vitamins C, B1, B2, B6, B12, folic acid, tocopherols and flavanoids, phenols, terpenes, and tannins. It also contains twenty mineral elements especially berry contains Al, As, Ca, Cd, Cr, Cu, Fe, K, Mg, Mn, Na, Ni, Pb, Rb and Zn. These substances also have effects on controlling arteriosclerosis, lowering the cholesterol level, anti-inflammatory, antimicrobial, pain relief, turning hyperthyroidism into euthyroidism and eliminating inflammation (Tigong, 1988). Furthermore, problems like indigestion, throat infection, gynecological problem, ulcer, bronchitis, acidity, diarrhoea, blood disorder, fever, gallstone, cough, cold, food poisoning etc. can also be cured. There are more than a hundred popular sea buckthorn-based formulations in various pharmacopoeias of *Sowa Rigpa* (Tibetan medicine). Seed oil contains vitamin K which promotes blood coagulation because of its catalytic role in forming prothrombin (Rongsen, 1992).Sea buckthorn leaf aqueous extract has significant anti-stress and adaptogenic activity (Saggu *et al.,* 2007). It was found to possess a very strong anti-viral activity and wide range of action against influenza and herpes viruses. It also showed inhibitory effect in a HIV infection in the cell culture and antimicrobial activity (Shuunguang and Chaode, 2001). The leaf extract also has a significant anti-dengue activity when evaluated in dengue virus Type 2 infected blood (Jain *et al.,* 2014).

Taxanomy and distribution

All the species of the genus Hippophae are native to Eurasia and are called sea buckthorns. It belong to Elaegnaceae, Order: Elaeagnales, Super order: Celastraneae, Subclass: Rosidae, Class: Magnoliopsida and Division: Magnoliophyta (Rajchal, 2009). Originally, it consisted of only one species, *H. rhamnoides,* with three sub species, rhamnoides, salicifolia and tibetiana (Servettaz 1908). The genus, *H. rhamnoides* has an extremely wide distribution throughout the Indian Himalayas but occur fragmentally in Europe and Asia (Zeb, 2004). In India, three different species of (*viz*. *Hippophae rhamnoides* L., *Hippophae salicifolia* D. Don and *Hippophae tibetana* Schultz.) are predominant species in the Himalayan regions. Of these, *Hippophae rhamnoides* L. is widely distributed along the Indus valley, and other valley of Ladakh including Nubra, Suru and Changthang whereas, *Hippophae tibetana* is widely distributed in Zanskar valley, Changthang valley. Similarly, *Hip pophae salicifolia* D. Don is also found in some pockets of North Sikkim, Upper Kinnaur, Lahaul and Kaza valleys of Himachal Pradesh, and in few hilly pockets of Uttrakhand as well.

Problems in breeding of sea buckthorn

Sea buckthorn is dioecious in nature, male and female plant of sea buckthorn cannot be determined until flowering, which usually takes place after 3–4 years of planting thus, can create hurdle in the breeding programmes as well as in its cultivation. Further, to improve more of its traits the availability of gene pool, restricted distribution of species and subspecies, unstable adaptability, lack of knowledge about extent of hybridization possible between species, non-availability of mapping populations, linkage maps and lack



Fig 1: Bright orange colour berries of sea buckthorn growing in wild.

of markers associated with important QTLs for quality characters may prove to be a major limitation in the identification of molecular markers for specific traits. Although, sea buckthorn found in Ladakh region have strong adaptability and fast growing, but botanically associated with many undesirable characters including small berry size, very soft skin (easily get rupture), thorniness of the bush makes harvesting process a tedious and labour intensive. Highly perishable nature of the berries and low pulp content makes it more difficult to handle after harvest. Vulnerability of the sea buckthorn genotypes to a multitude of biotic and abiotic stresses restricted their potential yield (Gupta *et al.*, 2000). At present, few biotic stress factors including diseases and insects pests affecting almost every stage/part of the sea buckthorn limiting its cultivation throughout the world. Among all the insect pests, carpenter moth (*Holocerus hippophaecolus*), a wood-boring pest has recently become a major threat to sea buckthorn in China (Zong *et al.,* 2008). The insect infestations reported from India include death hawk moth (*Acherontia styx*), defoliating beetles (*Brahmina* spp) and others such as Holotrichia longipennis and Plodia interpunctella which cause damage at early growth and fruiting stages (Li, 2003). In addition, damage to sea buckthorn is caused by deer, birds, rats and other rodents (Li, 2002). Similarly, the major fungal diseases reported on sea buckthorn include verticillium wilt, fusarium wilt, damping off, brown rot and scab. Since, sea buckthorn is a new cultivated crop, there are no registered pesticides or fungicides. Limited research related to the disease and pest control in sea buckthorn has been reported till date but can be controlled to some extent by physical, chemical and biological methods (Renjun *et al*., 2008). On the contrary, none of above insect, pest and disease infestations have been reported in the Trans-Himalayan region of Ladakh and it requires less management.

Achievements in sea buckthorn

At present, more than 60 cultivars have been described (Trajkovski and Jeppsson 1999). Worldwide, formal breeding programme on sea buckthorn was started in 1933 by Lisavenko Research Institute of Horticulture, Siberia. Efforts were made to improve local adaptation by crossing Russian varieties with Swedish collections, *H. rhamnoides* ssp. mongolica with Turkestanica of central Asian origin, the European ssp. rhamnoides with carpatica and caucasica from Asia Minor and utilized for breeding purposes (Bartish *et al*., 2000). The cultivated varieties from Russia and Mongolia show many promising agronomic traits, such as big fruits, few or no thorns, long fruit stalk, high content of bioactive substances and resistance to diseases, but show weak adaptability and are slow growing (Ruan and Li, 2005). Similarly, varieties from Russia and Mongolia have been introduced in China (since 1991) to improve qualities of presently growing cultivars as well as for enhancing their germplasm.

However, extensive survey was conducted in trans-Himalayan Ladakh for selection of high yielding genotypes. Major goals of a breeding programme is to develop thornless, large berry size, disease-resistant, fast growing varieties having strong adaptability and higher content of bioactive substances through conventional and molecular breeding so as to harness maximum economic gains from sea buckthorn. Consequently, plant breeders are motivated to study more of germplasm characterization, identification of genotypes as well as the extent of variability existing among the accessions. Germplasm characterization and evaluation is the first step in a breeding programme and inclusive information obtained from such an exercise would help breeders, geneticists and conservationists to effectively utilize the valuable genetic resources. Based on morphological markers of the plants various reports have been achieved like sex-related morphological and physiological responses in *H. rhamnoides*, reconstruct the relationship among the taxon of different regions to test association between morphological characters and environment, developed a set of morphological descriptors for the genus Hippophae after thorough study of the germplasm collected from different parts of India, morphological characteristics of pollen of ssp. caucasica in Turkey showed different features while pollens from Trabzon showed hybrid feature (Aras and Turkiyenin, 1995; Sabir *et al.,* 2003; Aras *et al*., 2007; Li *et al*., 2007; Mathew *et al.,* 2007). To overcome the limitations of morphological traits, biochemical and molecular markers have been developed. Biochemical markers like isoenzymes provided information on genetic variation, differentiation and evolution in seed of sea buckthorn (Yao and Tigerstedt, 1993), using SDS–PAGE, evaluated seed storage protein profile of cultivated and naturally grown plants in northern Pakistan and revealed the presence of only low molecular weight proteins in range of 15–50 kDa (Zeb and Malook, 2009), with using FAME examined biochemical relationships between genotypes of Eastern Anatolia (Ercisli *et al.,* 2008). Likewise, different molecular markers such as RAPD, SSRs, ISSR, AFLP, Chloroplast DNA, ITS sequences were successfully employed to study the genetic diversity, relationship among and within species of Hippophae, marker development for sex determination, phylogenetic relationship studies (Persson and Nybom, 1998; Bartish *et al*., 1999; Bartish *et al.,* 2000; Sun *et al*., 2003; Ruan *et al*., 2004; Ruan and Li, 2005; Sun *et al*., 2006; Sheng *et al.,* 2006; Singh *et al.,* 2006; Ruan, 2006; Ercisli *et al*., 2008; Wang *et al.,* 2008; Shah *et al*., 2009; Li *et al*., 2009; Ruan *et al*., 2009, Ding *et al.,* 2015). Recently, four ISSR markers have been developed associated with dried-shrink disease resistance in sea buckthorn which may prove useful in selecting resistant lines during breeding programs (Ruan *et al.,* 2009). Whereas, very limited information is available on microsatellite markers in sea buckthorn (Jain *et al*., 2010). It has been known for long that microsatellite variation sometimes leads to alteration in gene expression leading to phenotypic variation. Therefore, microsatellite variations could be implicated as a key player in the process of adaptation and evolution (Grover and Sharma 2011). In the recent past, many studies have been conducted on differential genes expression analysis using DeepSAGE in various plant species under stress conditions (Gyetvai *et al*., 2012; Kaminski *et al*., 2012). Since many genes are expected to mediate cold and freeze tolerance, a comprehensive study is required to identify cold and freeze

responsive genes in sea buckthorn using a high throughput sequencing technique such as DeepSAGE. Newly, 61 low temperature (LT) responsive extracellular proteins were reported from LT treated secretome in seabuckthorn (Gupta and Deswal, 2012), indicating the presence of a high number of cold responsive genes in sea buckthorn. Earlier, Ghangal *et al*. (2012) have identified some cold inducible elements through Expressed Sequence Tag (EST) based analysis in sea buckthorn.

To understand distribution of microsatellites in sea buckthorn transcriptome and assess their functional significance in developing Unigene Specific Microsatellite Markers (USMM). Jain *et al.,* 2013 reported unigenes of 7.69% harboured microsatellite repeats with an average of one microsatellite per 6.704 Kb transcriptome. Furthermore, microsatellites were densely populated in coding regions followed by 32 and 52 untranslated regions. AG and AAG type repeats were most frequently represented. Of the microsatellite positive unigenes, 48.81 % could be assigned gene ontology (GO) terms in order to assess associations between microsatellite containing unigenes and biological role of known genes. Utility of unigene specific microsatellites was assessed on the basis of polymorphism(s) detected in 18 sea buckthorn collections from Leh (India) using a set of randomly selected 25 unigene specific microsatellites.

Chawla *et al.,* 2014 determine early sex determination in sea buckthorn using sex linked SCAR markers HrX1 and HrX2 developed for *H. rhamnoides* were tested in *H. salicifolia and H. tibetana*. Result revealed that HrX1 produced female specific amplification in both *H. salicifolia* and *H. tibetana* while HrX2 did not amplify except for *H. rhamnoides*. Sequence based analysis of amplified SCAR marker products HrX1, HsX1 and HtX1 has shown homology with known plant acyl CoA ligases. Thus, the applicability of this single marker in all the three species has urged the need for de-novo development of sex linked markers in *H. salicifolia* and *H. tibetana*, so that saving of both time and resources can be accomplish.

The responses of sea buckthorn to cold stress were complex and involved numerous physiological, molecular and cellular adaptations. To assess the response during stress conditions He *et al.,* 2016 studied 2D-PAGE profile of sea buckthorn under LT stress. Result provides useful candidate proteins for genetic improvement and understanding the general ability of plants to respond to a wide range of external environmental stresses. In addition, mass spectrometry (MS) analysis identified 32 of 39 differentially expressed protein spots under low-temperature stress, and their functions were mainly involved in metabolism, photosynthesis, signal transduction, antioxidative systems and post-translational modification.

Ding *et al.,* 2016 utilize thirteen inter-simple sequence repeat (ISSR) primers to identify markers associated with oil content of dry pulp in 51 cultivars and lines of sea buckthorn, which clustered into three major groups based on 137 polymorphic markers. The oil content in dry pulp of 45 cultivars and lines in Group I ranged from 6.6 to 33.1% and these accessions belonged to *H. rhamnoides* ssp *mongolica* and its hybrids with *H. rhamnoides* ssp *sinensis*. Three lines (*H. rhamnoides* ssp *mongolica*) in Group II had high dry pulp oil contents (33.7 to 37.5%), whereas three lines of hybrids in Group III had low dry pulp oil contents (10.9 to 17.5%). The dry pulp oil content of *H. rhamnoides* ssp *mongolica* (27.2 ± 0.9%) was higher than that of hybrids (12.0±1.2%) ($P<0.01$). Four ISSR markers (881340, 8251000, 817380, and 8071100) had positive association with high dry pulp oil content ($P<0.01$) using stepwise multiple regression analysis. The use of these ISSR markers is a potential strategy to select genotypes with high dry pulp oil content and suitable parental combinations for improvement of sea buckthorn berries.

Puterova *et al.,* 2017 analyzed the transposable elements and satellites in its genome by utilizing Illumina DNA sequencing and reconstructed the main repetitive DNA sequences. They developed a new bioinformatics approach for advanced satellite DNA analysis and showed that about 25% of the genome consists of satellite DNA and about 24% is formed of transposable elements, dominated by Ty3/ Gypsy and Ty1/Copia LTR retrotransposons. Furthermore, FISH mapping revealed X chromosome-accumulated, Y chromosome-specific or both sex chromosomes-accumulated satellites but most satellites were found on autosomes. Transposable elements were located mostly in the sub telomeres of all chromosomes. The 5S rDNA and 45S rDNA were localized on one autosomal locus each. Although they demonstrated the small size of the Y chromosome of the sea buckthorn and accumulated satellite DNA there.

For the identification of sea buckthorn germplasm application of molecular markers (e.g., SSR and RAPD) to ensure sufficient genetic diversity was conducted by a (Lacis *et al.,* 2014). Six previously used polymorphic chloroplast SSR markers (cp SSR) were applied to characterize 33 accessions grown in Latvia, and developed from crosses among three subspecies of *H. rhamnoides*, namely ssp. *mongolica*, ssp. *rhamnoides* and *ssp. fluviatilis*. Although only three of the used cp SSR markers showed polymorphism in the investigated material, a grouping according to putative origin was achieved, and some samples with unknown origin were tentatively classified. In combination with eight SSR and sixteen RAPD markers, the cp SSR markers allowed complete discrimination of the tested sea buckthorn accessions, as well as determination of genetic structure in the Latvian sea buckthorn germplasm.

Recently, Nawaz *et al.,* 2018 applied twelve EST-SSRs (expressed sequence tags-simple sequence repeats) markers to differentiate genotypes in sea buckthorn. Significant differences in morphological traits were found across populations and between wild and village stands. A significant correlation was found between leaf area and altitude. Twenty-two color shades of berries and 20 dorsal and 15 ventral color shades of leaves were distinguished. Mean genetic diversity was comparatively high (H_e = 0.699). In total, three distinct genetic clusters were observed that corresponded to the populations' geographic locations. Considering high allelic richness and genetic diversity, the

Gilgit-Baltistan territory seems to be a promising source for selection of improved germplasm in sea buckthorn.

Achievements in biotechnology

Micro propagation is a fast method of plant propagation that has a great potential to develop high quality as well disease free plants. Advancement in this field have led to the development of several techniques for rapid multiplication and improvement of a wide range crops including sea buckthorn and their production systems. In sea buckthorn micro propagation includes two distinct types of *in vitro* differentiation, i.e. organogenesis and somatic embryogenesis have been used. Organogenesis has been induced through nodal segments, leaves, cotyledons, hypocotyls, apical meristem, roots from seedlings and axillary shoot production using explants from seedlings as well as from mature plants (Sriskandarajah and Lundquist, 2009; Chunhua *et al.,* 2000, Yang *et al*., 2004) and through embryogenesis using hypocotyls as explants (Liu *et al*., 2007; Sriskandarajah and Lundquist, 2009).

An efficient and rapid in vitro propagation protocol have been established using cotyledonary node explants excised from aseptically germinated seedlings of *Hippophae salicifolia* D. Don. Various nutrient media, antioxidants and different combinations of cytokinins and auxins were assessed and optimized for shoot proliferation (Saikia and Handique, 2014). Efficient rooting (100%) was achieved in a medium containing IBA (4.9 µM) on half-strength WPM. The rooted shoots showed 100% survival after acclimatization in the greenhouse. Furthermore, Random Amplified Polymorphic DNA (RAPD) fingerprinting profiles had been used to evaluate the genetic stability and clonal fidelity of the in vitro regenerated plants. Antibacterial activity was tested against six pathogenic strains by agar diffusion method.

Particle bombardment method for transforming sea buckthorn was tested by Sriskandarajah *et al.,* 2014. The early stages of induced adventitious shoots from roots were chosen as a novel target tissue for the transformation procedure. The root system was bombarded with gold particles coated with plasmid pRT99gus containing genes for plant kanamycin resistance and for β-glucuronidase expression, and shoots were regenerated under kanamycin selection. PCR analysis of the regenerated transformed lines confirmed the presence of a 603 bp gus (uidA) gene fragment, phenolics and a 1.5 kb fragment from the 35S promoter in three shoots from independent transformation events. Transient expression of the gus gene in roots or shoots after bombardment was weak, probably because of the high content of phenolics in the Hippophae tissue.

Institutes working on SBT in India

Potential of sea buckthorn has been recognized by several R&D organizations. Defence Research & Development Organisation (DRDO) has pioneered the sea buckthorn research in India and started several R&D projects since early nineties. Biotechnological potential has been recognized by Department of Biotechnology (DBT), Govt. of India, which initiated a project exclusively on sea buckthorn involving several R&D institutes and universities. Similarly, in the year 2008, Indian Council of Agriculture Research (ICAR) has approved a mega programme 'A Value Chain on sea buckthorn (*Hippophae* L.)' in which research institutes of ICAR, ICMR and NGOs are collaborative partners. Defence Institute of High Altitude Research, Leh and CSK HPKV, Palampur have initiated studies on developing packages and practices in Leh (Jammu & Kashmir) and Lahaul (Himachal). High yielding sea buckthorn selections are being identified and maintained at Defence Institute of High Altitude Research, Leh and CSK HPKV Palampur. Vegetative propagation is well established at DIHAR. A model orchard is established at DIHAR Leh. ICAR may undertake programs to develop sea buckthorn varieties suitable for cold desert conditions of Ladakh. A mission mode project for converting the vast barren land in Ladakh into green patch by planting sea buckthorn is ongoing.

CONCLUSION AND FUTURE PROSPECTS

Sea buckthorn is an economically and ecologically important plant species, currently being domesticated in various parts of the world including India. Unlike other horticultural crops, sea buckthorn in Ladakh holds a unique status with unmatchable adaptability with outstanding capacity to improve the environment and economic status of Ladakh. Due to its immense fruit qualities, and medicinal properties which led to unrestrained exploitation, therefore conservation and protection of its germplasm have become imperative. Efforts are being made to study phylogenetic relationship, genotypic characterization, using morphological, biochemical and molecular markers. Application of molecular markers for crop improvement has proved successful in crossbreeding and also in speeding up the pace of genetic improvement. However, molecular markers linked with QTL/ major genes for traits of interest and robust markers to determine the sex of the seedlings are need to be developed. Sea buckthorn is good source of freezing tolerant genes which further needs to be investigated. Furthermore, the New Generation Sequences are being utilizing to search for more valuable genes. Advances in biotechnological tools has provide further means of mass propagation of superior genotypes and generate new cultivars contributing in area expansion and enhances the productivity. However, there is need for optimization of the *in vitro* culture protocol as well as regeneration protocol for genetic transformation, mutagenesis to broaden up the genetic base of the crop.

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