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Morpho-molecular Characterization of local Genotypes of *Hyppophae rhamnoides* L. ssp. *Turkestanica* a Multipurpose Plant from Northern Areas of Pakistan

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Abstract: Seabuckthorn is one of the most important multipurpose plants having great value in medicines, health food, beverages, spray dried and freeze-dried powder, additives, cosmetics, as nitrogen fixer, fodder for livestock's and a tool for the control of soil erosion under degraded soil conditions. The plant can withstand extremes of the temperature ranging from 55°C(surface) to -43°C and grow well under drought conditions of about 250-800mm annual rainfall. It also withstands the soil pH from 5.8 to 9.5. Seabuckthorn thus can prove to be an effective plant for ecological purposes and economic activity for mountain communities. The present investigation was to identify the potential genotypes of Seabuckthorn for breeding better varieties in terms of fruit yield, fruit quality, nitrogen fixing ability and better adaptation in various agro-ecological zones of mountains of Pakistan. The initial investigation carried out at UCA Rawalakot included the collection of basic information regarding the cultivation and genetic diversity of the Seabuckthorn among the local genotypes based on morph-molecular tools consisting of phenotypic characters and the genotypic characters using SDS-PAGE. The phenotypic as well as genotypic studies revealed greater variation among the local genotypes with immense potential for future improvement using conventional breeding techniques. The general information revealed its potential to cultivate the plant directly without improvement under degraded soils of Azad Kashmir and elsewhere. However, in order to improve it's nutritional, medicinal and nitrogen fixing qualities and to breed thornless varieties for smooth harvesting, some conventional and non-conventional plant improvement techniques would be more meaningful.

Key words: Seabuckthorn, *Hyppophae rhamnoides* ssp. *Turkestanica*, genotypes, SDS-PAGE, multipurpose plant

Introduction

Seabuckthorn (known as a magic plant in China) belongs to Elaegnaceae family. There are six species in this family, among which the important species is *Hyppophae rhamnoides* L. which contains ten sub species. The only sub species found in the Northern Areas of Pakistan is *Turkestanica*. According to the worldwide distribution of *Hyppophae*, this sub sp. (*Hyppophae rhamnoides ssp turkestanica*) is widely found in the central Asia and the West Asia; that includes Afghanistan, Tajikistan, Turkmenistan, Uzbekistan, Kirghistan, Xinjiang province of China and Northern India. Northern Areas of Pakistan are just in the centre of the distribution of *H. rhamnoides* ssp. *Turkestanica* (Rongsen, 1996)

A typical Seabuckthorn plant usually consists of a bush bearing clusters of juicy fruits. The fruit is generally about the size of a small pea and is greenish in colour in the beginning but turns orange, red or yellow, as it matures. In composition, Seabuckthorn fruit is rich in nutrients, such as, carbohydrates, organic acids, amino acids and vitamins. Another feature of Seabuckthorn fruit is its oil; which is extracted both from the fruit and seeds. The oil contents ranges from 1.5-3.5% in fruit pulp to 9.9-19.5% in seeds. The oil has much more quantity of B-carotene and vitamin E therefore the oil of SBT is an effective medicine for many diseases. Fruit contains 60 to 80% juice in which sugar organic acids, amino acids and vitamins are rich. Vitamin C is 200 to 1500-mg/100g which is 5 to 100 times higher than any other fruit or vegetable known. Its leaves contain 11 to 22% crude protein, 3 to 6% of crude fat and some flavonoids (Wang Guoli, 1986; Rongsen, 1996). The fruit contains more than 100 types of nutrients and bioactive substances and more than 22 minerals. High quality wines, jams, jelly's, squash, powder juice, butter, ferments, tea and other healthful foods and syrups, are some main products, prepared form the fruit of Seabuckthorn (Rongsen, 1992; Shigri, 2001). Presently there are over 200 Seabuckthorn processing factories producing over 100 types of products in China (Rongsen, 1998).

Russians and Tibatens use to prepare drugs for various ailments have extensively used Seabuckthorn oil. The most important pharmacological functions of Seabuckthorn oils discovered by the scientists of the former USSR, were diminishing inflammation, disinfecting bacteria, relieving pain-promoting regeneration of tissues and for skin grafting, cosmetology and operational treatment of corneal wounds. (Anonymous, 2001). Many drugs based on Seabuckthorn products are registered and prepared in china like Seabuckthorn " Xindakang" a flavonide from the residues left from squeezed juice of fruits. The drug because of its potency in curing heart diseases is very well received internationally and its value was 8.0million US Dollars in 1996 (Rongsen, 1998). Other drugs in Chinese market are "Sweet Granule" used for treating cough, sputum, improves digestion and "Dried emulation" to improves brain function and brings about remission in case of memory loss (Rongsen 1992). Some other disease known to be treated by Seabuckthorn products are oedema, fever and chill, furuncle, abscess, obstruction by sputum, stomach tumor (Fuying and Tianming, 1989). According to the report of Pakistan International Acupuncture and Medical Sciences Lahore, 30-40 persons having different diseases of hepatitis, cancer and ulcer were treated successfully with Seabuckthorn oil (Shigri, 2001).

Seabuckthorn is also important as fuel wood, fencing, fodder, and plant protector, to make soil fertile and also used for the purposes of shelter. It has been reported that a five years old plant can check 90% run-off water and 95% soil erosion. The newly emerged soft twigs and leaves are used as fodder for animals. An adult Seabuckthorn plant can withstand temperature ranging from 55 to -43°C and can grow well at pH range from 5.5-9.5. It can withstand atmospheric drought at an annual rainfall of 250-800mm (Rongsen, 1992). Having high nutritional value in leaves and small twigs, the plant provides favourable environment and feed for 29 kinds of wild animals and 51 kinds of birds in China. The plant have very strong root system, five years old plant has taproots of up to 1.1m deep and horizontal roots of up to 2.58m wide. Lot of seedling grows upwards from the horizontal roots and form new bushes. A symbiotic micorhizal fungus, Frankia, has been found on Seabuckthorn roots, which form nodules and fixes maximum amount of nitrogen present in the atmosphere, its capacity to fix nitrogen is twice than that of soybean (Rongsen, 1992).

Morphological characterization is a conventional techniques used for evaluating the plant diversity as a tool for breeding and improvement. Molecular/Biochemical techniques are new but are routinely used these days for determining the genetic variability among the species, genotypes and the populations (Moller and Spoor, 1993; Ashour *et al.*, 1995). Waines and Payne (1987)

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analyzed glutenin through SDS-PAGE in the A genome of 497 diploid wheats and in 851 landraces of bread wheat, in which 4 races with HMW sub-units were discovered. Ciaffi *et al.* (1993) used SDS-PAGE by studying 315 populations, in which total 44 different banding patterns were identified. The technique is quick and accurate to identify the variability in natural populations of the plants, especially to evaluate the wild germplasm for its use in the development of economically viable varieties. Domestication of sea buckthorn started in Siberia in the 1930s (Kalinina and Panteleyeva, 1987). Local germplasm (ssp. *Mongolica*) from the Altai Mountains was used in the onset of the breeding. Breeding projects have, later on, been initiated also in other countries such as Germany (Albrecht, 1990), Finland (Yao and Tigerstedt, 1994), China (Huang, 1995) and Canada (Li and Schroeder, 1996). At SLU-Balsgård, Sweden, breeding of sea buckthorn started in 1986. Conventional breeding methods, including germplasm evaluation, hybridization and selection, are used (Trajkovski and Jeppsson, 1999).

The mountainous regions of Pakistan including Azad Kashmir and Northern areas are at high risk of erosion because of the loss of forests and changing weather. The land holdings in this region are very small and the economic conditions of the farmer are very poor. Reforestation of pines and other slow growing plants is generally not a suitable option. Poverty stricken peasants are dependent on land for farming and reclaim land by destroying the remaining forest. As land productivity is low more land is being reclaimed. This vicious cycle leads to progressive land degradation. The activity is rendering the loss of many valuable resources of the area including the genetic diversity and the top cultivable fertile layer of soil apart from the loss of storage water in Mangla and Terbela reservoirs. The use of quickly growing vegetation has been perceived as a major and most promising tool to control land degradation in any part of the land especially in mountains (Ahmad and Chauhdry, 1995). Seabuckthorn is one of the species successfully used on a large scale, particularly in Northern China to control decertification, to conserve land and water resources, and to integrate economic exploitation with ecological rehabilitation. The plant can be used for the ecological and economic development of mountainous regions of Pakistan including Northern Areas and Azad Jammu and Kashmir. The objectives of the present investigation were:

- To familiarize this multipurpose plant among the local communities for ecological and economic development of the area.
- To evaluate the genetic diversity among genotypes and land races of Seabuckthorn from Northern Areas of Pakistan using conventional and molecular techniques for the development of suitable varieties.

Materials and Methods

The investigation was carried out on the four genotypes of seabuckthorn sub sp. *Turkestanica* found in Baltistan during the year 2001. The four genotypes were designated as SBT-01, SBT-02, SBT-03 and SBT-04 on the basis of fruit morphology I-e colour and size of the fruits (large size with red colour SBT-01; small size with red colour SBT-02; large size with yellow colour SBT-03; small size with yellow colour SBT-04). The genotypes were investigated for morphological and genetic diversity. Five comparable plants from five different localities were randomly selected for each genotype. The average values were calculated for each character using different replications.

Morphological characters investigated were, plant height, number of main branches per plant, number of sub branches per main branch of plant, number of thorns on main branch of each plant, number of fruits on main branch of each plant, plant canopy and girth of the stem of each genotype. The data was analyzed using computer based statistical programme.

Extracting the total seed proteins from the genotypes compared and fragmenting them in SDS-PAGE carried out Molecular/Biochemical investigations. Total seed proteins were extracted by using an established method routinely used in Plant Breeding and Genetics Laboratory of UCA-Rawalakot (Ahmad *et al.*, 2000). Preparation and polymerization of gels and electrophoresis was carried out by a little modification in the standard method given by Laemmli (1970). Photograph of the gels were taken after staining and destaining the gels for reference. The distance covered by different protein bands were closely visualized on a light box, the pattern was also drawn on a paper for later reference. The comparisons were made between the common and variable protein-banding pattern of four genotypes.

Results

Comparisons based on morphological characters indicated larger amount of variability among the genotypes compared. The plant height although was variable in different genotypes but was not found to be significant statistically ($P>0.05$). The ranking order in plant height was SBT-01, SBT-03, SBT-04 and SBT-02 respectively. Similarly the characters like the plant canopy were

Table 1: Number of branches/plant

SOV	df	SS	MS	F.CAL.	F.TAB.
Treatment	3	10.95	3.65	4.25**	3.26**
Replication	4	1.30	0.325		5.41*
Error	12	10.30	0.858		

Table 2: Number of sub branches/plant

SOV	df	SS	MS	F.CAL.	F.TAB.
Treatment	3	765.2	255.066	12.737*	3.26**
Replication	4	47.7	11.925		5.41*
Error	12	240.3	20.025		

Table 3: Stem girth (cm)

SOV	df	SS	MS	F.CAL.	F.TAB.
Treatment	3	26.5375	8.8458	11.197*	3.26**
Replication	4	12.23	3.0575		5.41*
Error	12	9.49	0.79		

Table 4: Number of berries/main branch

SOV	df	SS	MS	F.CAL.	F.TAB.
Treatment	3	765.2	255.066	12.737*	3.26**
Replication	4	47.7	11.925		5.41*
Error	12	240.3	20.025		

Table 5: Number of thorns/main branch

SOV	df	SS	MS	F.CAL.	F.TAB.
Treatment	3	855.35	285.11	11.22*	3.26**
Replication	4	89.5	22.375		5.41*
Error	12	304.9	25.408		

* = $P<0.01$, ** = $P<0.05$

Table 6: Comparison of banding pattern (total seed proteins) in SDS-PAGE for 4 genotypes of *Hyppophae rahmnoides* ssp. *Turkestanica*

Distance travelled (cm)	SBT-01	SBT-02	SBT-03	SBT-04
0.5				
1.0		+	+	
1.5	+	+		
2.0				
2.5				
3.0	+			
3.5		+		
4.0		+	+	
4.5		+	+	
5.0		+	+	+
5.5	+	+	+	+
6.0	+	+		
6.5	+		+	+
7.0		+		

quite variable but did not show significant difference among the genotypes compared ($P > 0.05$). The ranking order regarding plant canopy however, was found to be SBT-04, SBT-03, SBT-01 and SBT-02 respectively. Comparisons between the numbers of braches per plant among different genotypes indicate significant difference among the genotypes ($p < 0.05$) (Table 1, Fig.1). When number of sub-branches/ main branch of each genotype was compared (Fig. 2, Table 2), the difference was found to be highly significant ($p < 0.01$). Similar results were obtained ($p < 0.01$) when girth of plant was compared among the genotypes (Table 3, Fig. 3). The characters like the number of barriers/main branch from each genotype and the number of thorns/ main branch from each genotype were compared, highly significant differences were found ($p < 0.01$) in these characters among the Seabuckthorn genotypes compared (Table 4, 5, Fig. 4, 5). Likewise the fruit colour and fruit size was also found to be variable in different genotypes compared. In order to see the variability at the level of genes or the gene products (seed Proteins), comparisons were made by the fractionation of total seed proteins in SDS-PAGE.

Protein was extracted from the seeds of four genotypes compared. The gels after electrophoresis was stained with coomassie brilliant blue and photographs were taken for reference. In the absence of standard protein markers, comparisons among different genotypes were made on the basis of banding pattern or electrophoretic mobility with in the gel. The banding pattern was also drawn on white paper with the help of a measuring ruler. The electrophoretic mobility of total seed proteins among four genotypes of *Hyppophae rhamnoides* ssp. *Turkestanica* indicated significant variability in the mobility of these proteins among the genotypes compared (Table 6).

In the genotype, SBT-01, 5 bands were found at the distances of 1.5, 3, 5.3, 6, and 6.3 cm. In SBT-02, 7 bands were at the distances of 1.5, 3.5, 4, 4.6, 5.3, 5.8 and 6.8cm. Whereas in SBT-03, 6 bands at the distances of 1, 4, 4.5, 4.9, 5.4 and 6.3cm were observed and in SBT-04, 5 bands at the distances of 0.9, 4.3, 4.6, 5.1 and 6.1cm were observed. Only one band at 5.5cm was found to be common in all the genotypes compared. In the genotypes SBT-01 and SBT-02, two bands were common at 1.5 and 5.3cm apart from a shared band in all genotypes. In SBT-01 and SBT-03 one band at 6.3cm was common, but in SBT-01 and SBT-04 no band was common except the one shred by all genotypes. In the genotypes SBT-02 and SBT-03 two bands at 4 and 5cm were common while in SBT-04 a single band at 5cm was observed in common to SBT-02 apart from the common band of 5.5cm shared by all genotypes. In the genotypes SBT-03 and SBT-04, maximum bands (3 bands) were shared at 1cm, 4.5 and 5cm apart from the common band of 5.5cm shared by all genotypes compared. The results therefore, indicated that the genotypes SBT-03 and SBT-04 were closely related followed by SBT-3 and SBT-2. The genotypes SBT-01 was least related with SBT-4 but showed some relationship with SBT-2 and SBT-3 as indicated by SDS-PAGE fractionation of seed proteins.

Discussion

Morphological as well as the genetical investigations were carried out to determine the variability that exists among the different genotypes of seabuckthorn sub sp. *Turkestanica*. The genetic diversity in the genotypes of the sub sp. *Turkestanica* would mean the variability in the chemical constituents of various natures including nutritional, medicinal and nitrogen fixing ability of a multipurpose plant.

Morphological characters including number of main branches per plant, number of sub-branches per main branch of plant, number of barriers per main branch, number of thorn per main branch and plant girth were investigated to see whether any diversity exists amongst the genotype. The results were highly significant ($p < 0.01$) in most of the characters compared among the genotypes except the plant height and plant canopy (Tables 1, 5, Fig. 1, 5). It would mean that immense variation exists among the natural genotypes

of Seabuckthorn, which are indicative of the variability in the genotypes found under somewhat similar environmental conditions. The variation therefore, may not be due to

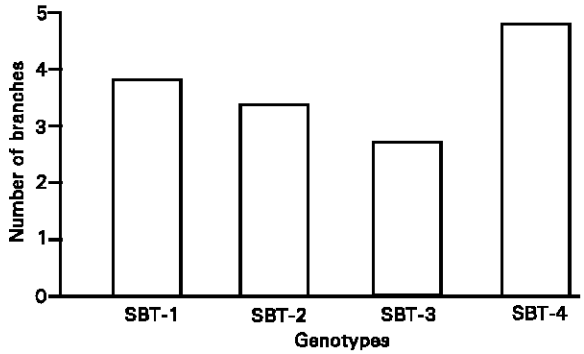

Fig.1: Number of main branches/plant in SBT genotypes compared SBT-1 =3.6, SBT-2=3, SBT-3=2.4, SBT-4=4.4

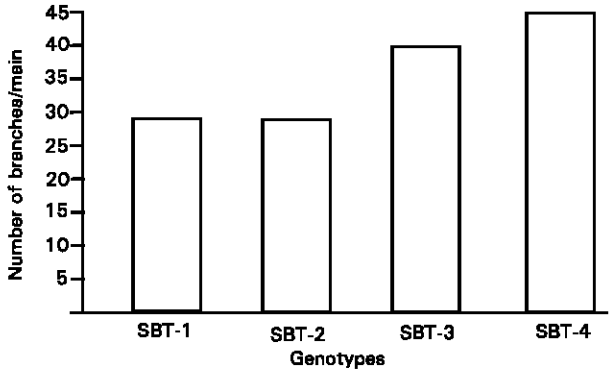

Fig.2: No of sub-branches/main branch of SBT genotypes compared SBT-1 =30.4, SBT-2=25.8, SBT-3=37, SBT-4=42

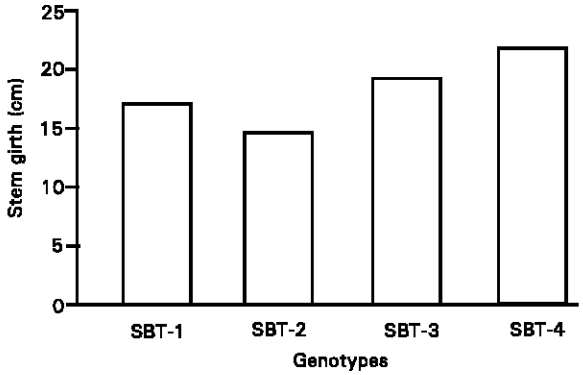

Fig. 3: Main stem girth (cm) in SBT genotypes compared SBT-1 =15, SBT-2=12.5, SBT-3=17.5, SBT-4=20.25

environment only. Similar results have been indicated among the genotypes of Seabuckthorn in Chinese part. It has also been indicated that the phenotypic characters like fruit size and colour may not be the indication of genetic variability always (Rongsen, 1992). In order to see the variability at genetic level SDS-PAGE banding pattern of the gel using total seed protein was also investigated.

When total seed proteins extracted from the seeds of different genotypes were compared (Table 6), diversity in most of the bands was indicated by their movement in the gel. Only few bands were of similar distances but most of the bands were of variable size. Such seed proteins variability in SDS-PAGE is very frequently used to identify the genotypes/populations of various

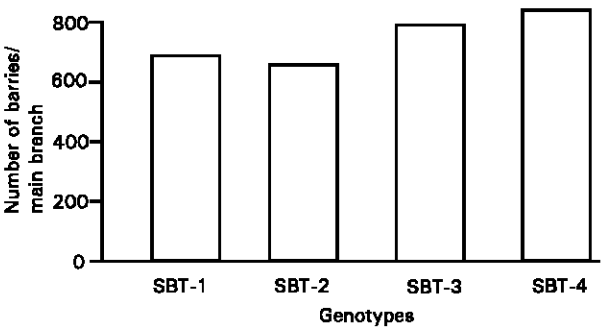

Fig. 4: No. of barries/main branch of plant in SBT genotypes compared, SBT-1 = 642.6, SBT-2 = 584.6, SBT-3 = 711.4, SBT-4 = 756.4

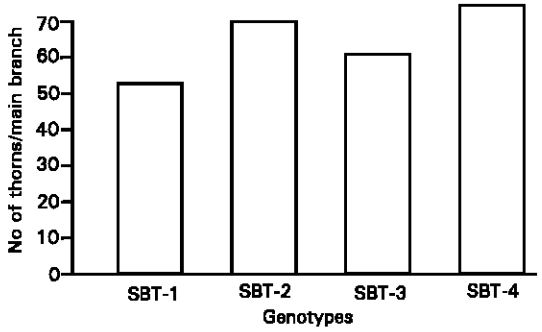

Fig. 5: No. of thorns/main branch in SBT genotypes compared, SBT-1 = 49.4, SBT-2 = 64.4, SBT-3 = 56.4, SBT-4 = 66.8

plant species (Yong *et al.*, 1995; Ciaffi *et al.*, 1993). Ashour *et al.* (1995) investigated the four-ascarded nematodes by using SDS-PAGE and concluded that *T. vitulorum* was the most divergent species of the four that was studied. Moller and Spoor (1993) suggested that discrimination and identification of *Lolium species* and cultivars is possible by rapid SDS-PAGE of seed storage proteins. Similar results have been reported by Waines and Payne (1987) by analyzing glutenin through SDS-PAGE in the A genome of 497 diploid wheat and in 851 landraces of bread wheat, in which 4 races with HMW sub-units were discovered.

The bands of proteins in different Seabuckthorn genotypes at the same distances depicted genotypic similarity. At the distances of 5.3 and 1.5cm in the gel, the genotypes SBT-01 and SBT-02, shared two common bands, where other two genotypes (SBT-03 & SBT-04) expressed no band at these particular distances. When these results were compared with phenotypic observation, it was found that these two genotypes had similarity in number of branches per plant (3.6 and 3 respectively) as well. Similarly the genotypes SBT-02 and SBT-03, shared a common band at the distance of 4cm, in the gel. These two genotypes also showed similarity up to a considerable extent in the number of thorns/main branch of plant (62.4, 56.4 respectively) and number of main branches per plant (3, 2.4 respectively).

In the same manner, genotypes SBT-02 and SBT-04 have a common band at the distance of 4.6cm, in the banding pattern of the gel. These two genotypes also expressed similarity in the parameters, plant height (212.5, 220cm) and no of thorns per main branch of plant (62.4, 66.8 respectively). The similarity in certain phenotypic characters and the commonality of 3 protein bands in SBT-03 and SBT-04 was also observed apart from the variability in these characters. It would mean that the genotypes of *Hyppophae rhmnoides* ssp. *Turkestanica* investigated have common lineage but diverged during the period of their adaptation by natural selection and could provide good stock for future breeding programme.

The investigation proved the affectivity and the efficiency of SDS-PAGE technique in analyzing the natural germplasm of Seabuckthorn. The results indicated the importance of the natural germplasm of Seabuckthorn, which need to be included in any programme to develop suitable varieties of the plant for economic activity and commercial exploitation in mountainous regions of Pakistan. The work was first of its kind in Pakistan, however these preliminary results are demanding further investigation to enhance the quality and production of such valuable plants and their commercial products.

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