



Cultivation, Diversity and Bio-Potential of *Salix* in the Kashmir Himalaya, India

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ABSTRACT

Represented by about 450 species in the world, genus *Salix* (Salicaceae) is mainly distributed in the temperate regions of the Northern Hemisphere. *Salix* has been regarded as a platinum crop worldwide and presently its production on large scale is meant for best possible return in yield and profit. *Salix* plays a dominant role in shaping economy of the State of Jammu & Kashmir as it provides raw material for handicrafts, horticulture, sports, ply board industries besides, providing fodder for cattle and sheep and also utilized as fire wood. Keeping in view its importance, various *Salix* species/cultivars were cultivated for three consecutive years under Short Rotation Coppice (SRC) method to record the growth in various attributes (leaf size, no. of branches per cutting, length of branches and no. of leaves per branch). Target accessions showed increase in biomass in all the attributes which was highest in the third year of cultivation. DNA finger printing technique- Amplified Fragment Length Polymorphism (AFLP) applied to characterize and understand the genetic relationship, revealed prominent diversity among studied species/cultivars of *Salix*. This study is imperative in developing a standard protocol for optimal utilization of abundant willow resources of the State for maximum biomass production in order to efficiently sustain the supply of raw material to potential industries for the manufacture of bio-fuels, bio-energy, bio-medicine, bio-crafts etc.

Key words: *Salix*, biomass, handicraft, genetic diversity, bio-medicine.

INTRODUCTION

Represented by about 450 species in the world, genus *Salix* (Salicaceae) is mainly distributed in the temperate regions of the Northern Hemisphere (Argus, 1999). Family Salicaceae *sensu stricto* is taxonomically complex which traditionally comprises of three genera - *Salix*, *Populus* and *Chosenia* with more than 200 species in the world (Rehder, 1940). However, recent studies have revealed that this family includes 1220 species spread over 56 genera (Christenhusz, 2016, Stevens, 2008). Genus *Salix* includes willows, also called

sallows, and osiers, includes deciduous trees and shrubs, found primarily on moist soils. *Salix* has very rich distribution in Kashmir Himalaya. Lambert (1933) reported 09 species of *Salix* from Kashmir. Chatterjee (1939) reported 28 species of family *Salicaceae* from Kashmir Himalaya. 15 and 21 species of *Salix* have been cited from Kashmir Himalaya (Javeid, 1972 and Stewart, 1972).

Salix is regarded as a 'Platinum Crop' across the world as it has remarkable potential and economic significance. Its species can be cultivated and raised very easily by cuttings and hence retaining all characteristics of the plant besides producing large quantities of the original plant material. In Kashmir, majority of *Salix* species have been introduced and propagated from cuttings - *S. triandra* L., *S. alba* L., ssp. *coerulea* (Sm). Rech., *S. purpurea* L. being the common ones. *S. alba* L. ssp. *coerulea* produce the best quality cricket bats while *S.viminalis* is the important wicker willow (Javeid, 1972).

Salix is a vital bio-medicinal crop as its bark and leaves contain substantial amounts of Salicylic acid which is the original source of aspirin which has antipyretic and analgesic properties. It is used as an alternative crop for the production of wood-fuel. It is an efficient renewable energy crop which has higher productivity and lower production costs. Hence, it is widely used as bio-energy and bio-fuels. Different *Salix* species are commonly used for phytoremediation due to their rapid growth rate, ease of propagation and contaminant uptake capability (Pulford et al. 2002).

The economy of the State of Jammu and Kashmir is shaped by willows. They play a dominant role by providing raw material for handicraft, traditionally used in wickering articles for daily use and work of art, horticulture industry, sports (cricket bat) industry, match factories, plywood and ply board industries, fodder for sheep and cattle population and serves as source of fire wood.

In this backdrop, present study was undertaken to resolve the taxonomic complexity in *Salix* by designing a research programme for carrying out detailed investigation on various economically important *Salix* species growing in the Kashmir Himalaya. Thrust was to develop a standard protocol for optimal utilization of abundant willow resources of the State for maximum biomass production in order to efficiently sustain the

supply of raw material to potential industries for the manufacture of bio-fuels, bio-energy, bio-medicine, bio-crafts etc. The objective of this work was to cultivate various *Salix* species/cultivars as per the internationally accepted protocols and to characterize and study the diversity among them through AFLP technique.

MATERIALS AND METHODS

Salix species were collected from different sites of the State, identified and then cultivated for three consecutive years under Short Rotation Coppice (SRC) (Danfors et al. 1998) with slight modifications in length of cuttings and spacing between rows as well as between cuttings.

2.1 Cultivation

- Manual weed removal from the planting site was done.
- Deep ploughing of soil and addition of organic manure was carried out.
- Basic fertilizers in the form of phosphorous, potassium and nitrogen were supplied.

2.2 Propagation

- 24 inches long cuttings of different *Salix* species were taken for planting.
- Cuttings were inserted into the soil, 20 inches above and 4 inches below ground.
- 6 inches was the distance kept between the cuttings as well as between the rows.
- Sufficient water was made available to the cuttings.
- Pruning at the stump height of 5-10 cm (coppicing) was done after one year of propagation.

In the present study, seventeen collected accessions were grown, maintained and propagated for three consecutive years in Kashmir University Botanical Garden (KUBG). Growth attributes taken into consideration for the present study include size of the leaves (length and breadth), number of branches per cutting, length of the branches and number of leaves per branch. Data pertaining to these attributes was collected on monthly basis. All measurements were done randomly and mean was calculated.

2.3 AFLP analysis

Amplified Fragment Length Polymorphism (AFLP) analysis was carried out in order to estimate the diversity within different *Salix* species/cultivars.

2.3.1 DNA isolation

For DNA extraction, young *Salix* leaves were collected and lyophilized (in liquid nitrogen at -196°C). 70-75 mg of freeze dried leaf sample was ground to a fine powder in pestle and mortar using liquid nitrogen. Ground tissue was transferred to centrifuge tube to which 400 μl of lysis buffer and 4 μl of RNase was added. Thorough mixing was done and then it was incubated for 10 minutes at 65°C . 150 μl of precipitate buffer was added and centrifugation of the lysate at 15000 rpm was done for five minutes. Flow-through fraction was transferred to a new tube without disturbing the cell debris pellet. 400 μl of precipitation buffer was added to the lysate and mixed gently which was then transferred to mini spin columns (binding columns) and centrifuged for one minute at 8000 rpm. Flow through fraction from the above step was discarded and 500 μl of wash buffer (70% ethanol) was added to the retained fraction. 100 μl of elution buffer was added, followed by centrifugation at 8000 rpm for one minute to elute. AFLP analysis was carried out according to procedure described by Vos et al. 1995.

Each sample consisting of approximately 200ng DNA was digested with EcoRI/MseI restriction enzyme solution. The reaction mixture was diluted 10 fold with TE buffer. 20 and 23 cycles of pre-amplification and selective amplification reactions were carried out. The pre amplification reaction product was diluted in the ratio of 1:50. 6% polyacrylamide gels containing acrylamide and bisacrylamide in the ratio of 29:1, 7.5M urea and 1xTBE buffer was used for separation of amplification products. The electrophoresis of gels was carried out at constant temperature and wattage for about 2.5 hours with 1xTBE. Silver staining process was used for the visualization of the gels with a transilluminator.

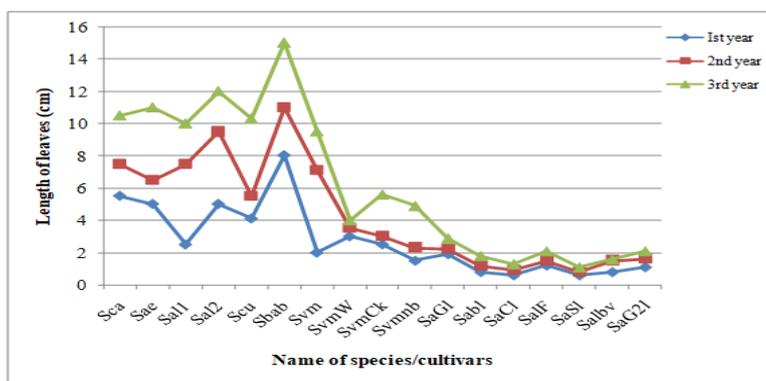


Fig. 1: Length of leaves of different *Salix* species/cultivars for three consecutive years.

2.3.2 Data analysis

Scoring of polymorphic bands was done manually with presence as 1 and absence as 0. Compilation of the data was carried out by using NTSYSpc (Numerical Taxonomy and multivariate analysis System) software version 2.0 (Exeter Software, New York) (Rohlf, 1990). The analysis was also used for phylogenetic tree estimation, which was visualized as a graphical dendrogram.

Various symbols used in figs and dendrogram to describe different species and cultivars of *Salix* are described as: Sca- *Salix caprea*; Sae- *Salix aegyptica*; Sal1- *Salix alba*; Sal2- *Salix* sp.; Scu- *Salix matsudana*; Sbab- *Salix babylonica*; Svm- *Salix viminalis*; SvmW- *Salix viminalis* (white); SvmCk- *Salix viminalis* (red); Svmnb- *Salix viminalis* (black); SaGl- *Salix alba coerulea*; Sabl- *Salix alba* (Ladakh); SaCl- *Salix fragilis*; SaFl- *Salix wallichiana*; SaSl- *Salix pycnostachya*; Salbv- *Salix alba micans*; SaG2l- *Salix daphnoides*.

RESULTS

Short Rotation Coppice (SRC) method of cultivation protocol has been found very valuable for raising biomass in *Salix* species/cultivars. The overall growth showed an increase in biomass which was maximum in the third year of establishment. Coppicing (pruning at stump height of 5-10 cm) also produced highest number of shoots which subsequently yielded maximum biomass.

Marked variations were recorded for leaf size during the three years of cultivation. Maximum length (15 cm) of leaves was recorded in *Salix babylonica* while as minimum leaf length (1.1 cm) was recorded in *Salix*

pycnostachya (Fig. 1). *Salix alba* ssp. *micans* and *Salix viminalis* (black) showed maximum and minimum leaf breadth of 11.9 cm and 1.1 cm respectively (Fig. 2).

Data recorded for number of branches per cutting in different *Salix* species under cultivation for three consecutive years showed a maximum of 41 branches in *Salix* sp. and *Salix viminalis* (white) while a minimum of 5.3 branches were noted in *Salix wallichiana* (Fig. 3). In the third year of cultivation, the maximum and minimum length of branches of 75 cm and 11.8 cm was

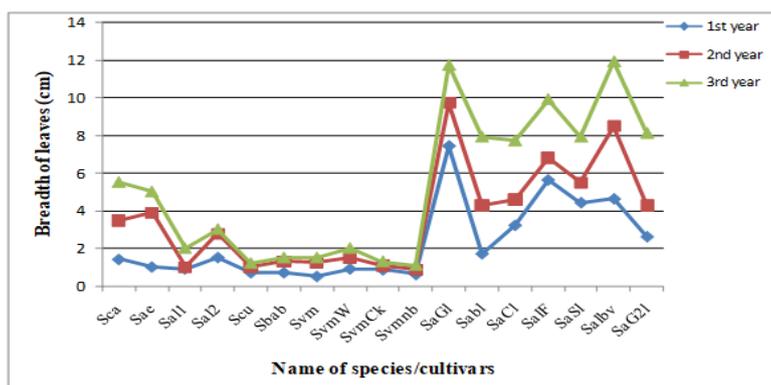


Fig. 2: Breadth of leaves of different *Salix* species/cultivars for three consecutive years

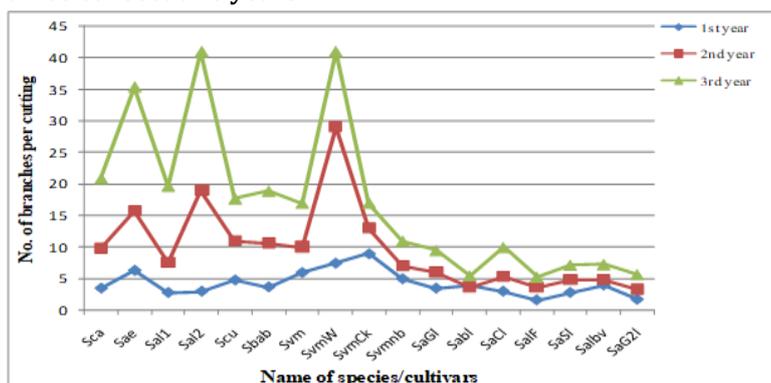


Fig. 3: Number of branches per cutting of different *Salix* species/cultivars for three consecutive years.

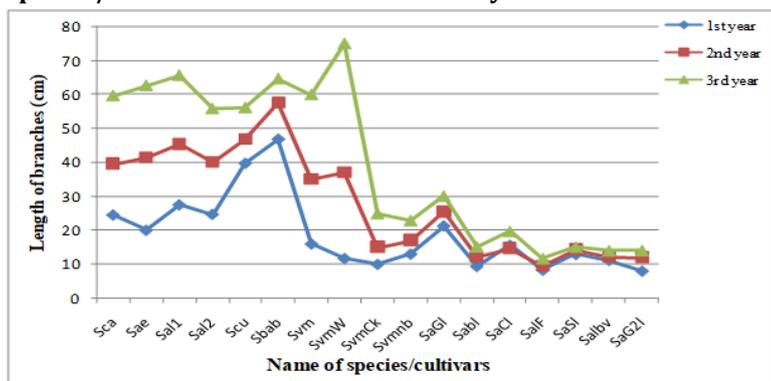


Fig. 4: Length of branches of different *Salix* species/cultivars for three consecutive years

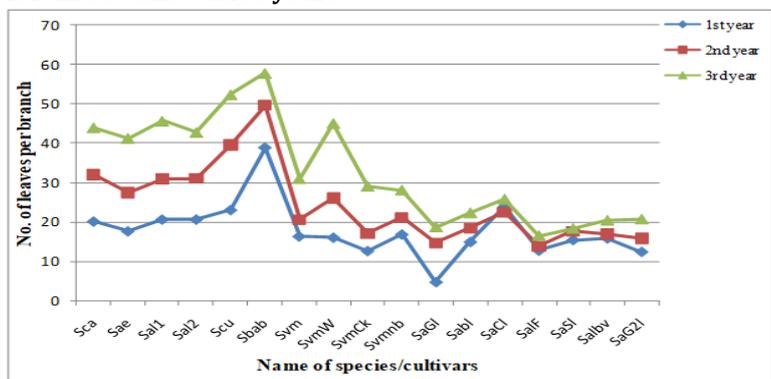


Fig. 5: Number of leaves per branch of different *Salix* species/cultivars for three consecutive years.

observed in *Salix viminalis* (white) and *Salix wallichiana* respectively (Fig. 4). The number of leaves per branch also showed an increase during the three years of cultivation. In the last year of cultivation, a maximum and a minimum number of 57.8 and 16.4 leaves per branch were recorded in *Salix babylonica* and *Salix wallichiana* respectively (Fig. 5).

Five EcoRI/MseI primer combinations used during the present study generated a total of 4518 polymorphic bands. The average percent polymorphism was 83.11% from the five primer combinations employed for this study. The potential of different primers to generate polymorphic AFLP markers varied significantly over all genotypes, thereby confirming the high multiplex ratio expected from the AFLP technique. The most diverse genotypes as revealed by AFLP analysis were *Salix alba* subsp. *micans* and *Salix viminalis* (red) since they shared the least similarity value of only 0.33. Dendrogram was constructed using UPGMA (Unweighed Pair Group Method Arithmetic mean) analysis (Fig. 6). The resulting dendrogram provides an excellent estimate of the phylogeny and diversity of different *Salix* species/cultivars.

However, as revealed from the study, morphologically there is lot of similarity between some of the cultivars but, when these cultivars were analyzed using AFLP technique, conspicuous differences were recorded. AFLP results confirmed that *Salix* genotypes studied are distinct from one another as is evident from the large genetic distances between them.

DISCUSSION AND CONCLUSION

Short Rotation Coppice (SRC) method of cultivation (Danfors et al. 1998) applied to all *Salix* species/cultivars selected for the present study has been found very useful for raising biomass for research oriented activities like bio-fuels, bio-energy, bio-medicines, bio-crafts etc. Maximum yield was obtained at the closest spacing. The observation was the same as reported by

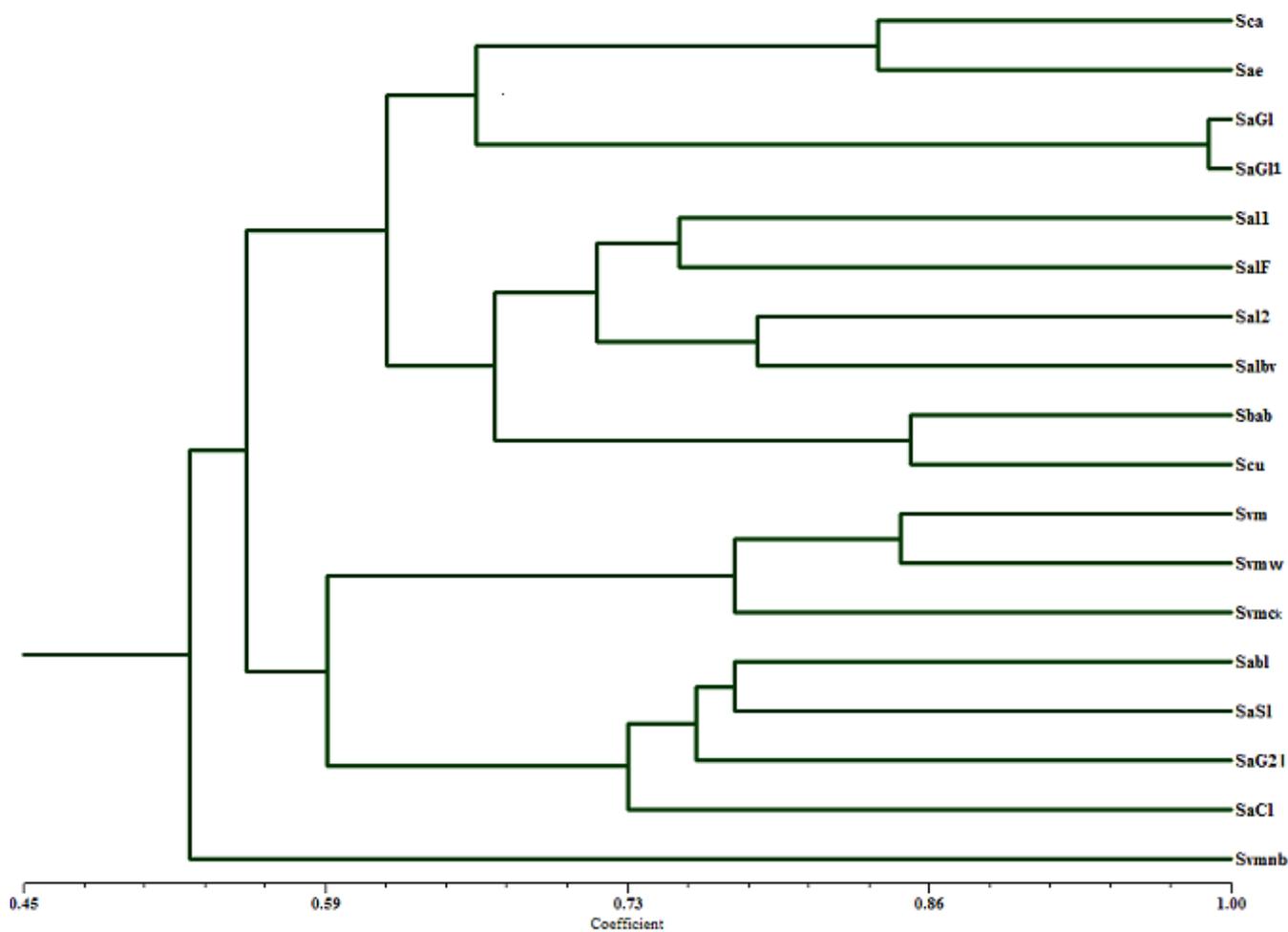


Fig. 6: Dendrogram showing diversity of 17 *Salix* species/cultivar

Rushton, (1998) for spacing of willows grown as Short Rotation Coppice in Sweden. Similar type of study (Christerson *et al.* 1994) was carried out using intensive short rotation coppice cultivation of *Salix* (short rotation forestry) on agricultural land for the production of biomass for energy and fiber and its integration with environmental aspects such as bio-filters, waste recycling, and utilization of sludge as a fertilizer. Willows (*Salix* spp.) grown as short rotation coppice (SRC) are among the most important woody bioenergy crops of northern temperate regions (Hanley, 2014 & Karp *et al.* 2011).

The growth during the three consecutive years showed an increase in all the attributes which was maximum during the third year of establishment. In a similar study (Abrahamson, 1998) it was observed that biomass for bio-energy based on willow, planted and managed at high densities and short (3-4 year) coppice harvest cycles, provide fuel for co-firing with coal and have environmentally and ecologically sustainable production and utilization systems.

Amplified Fragment Length Polymorphism (AFLP) analysis have proved useful for estimating diversity particularly to assist in the conservation of rare species and plant genetic resources. The data on relationships and variability within the population of some economically important species of *Salix* based on AFLP markers have been worked out in the present study as it has been effectively used to fingerprint willows for genetic distance estimates (Barker *et al.* 1999). The study was carried out on the same concept for the first time and AFLP markers were used to identify *Salix* species/cultivars that represent the genetic variation. All of the primer pairs applied in this study revealed high levels of polymorphism, confirming that high diversity exists within the *Salix* genome. In a related study (Angaji *et al.* 2012; Sulima and Przyborowski, 2013) for genetic variation in *Salix* random markers were used which showed high degree of polymorphism.

150 polymorphic bands make it possible for a researcher to reliably estimate genetic similarities

among genotypes within the same species (Pejic *et al.* 1998). In confirmation to this, it was found that with five primer pairs, generating 4518 polymorphic bands, it was possible to fingerprint all of the 17 species/cultivars included in this study.

The number of polymorphic bands necessary to accurately estimate similarity among willows should be relatively low because of the broad geographic range from which parents were selected and the high degree of variation that exists in this species (Kopp *et al.* 2002). Results of AFLP fingerprinting in this study suggest that there was in general, large diversity among different *Salix* species/cultivars and AFLP fingerprinting can substantiate in developing an important tool for their rapid identification and establishing correct nomenclature for raising biomass used as a potential resource in various thrust areas like, bio-energy, bio-fuels, bio-medicines and bio-crafts etc.

Conflicts of interest: The authors stated that no conflicts of interest.

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