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RESEARCH ARTICLE

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GENOTYPIC VARIATIONS FOR SALINE TOLERANCE IN MORUS SPECIES BASED ON THEIR OVERALL ATTRIBUTES

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ABSTRACT

A study to determine the nutritive value of mulberry (*Morus sps.,)* leaves, under the saline stress condition was conducted. Here four major saline tolerant genotypes of mulberry (S1635, S36, S13, and MR2) were taken for the test, and their nutritional values were estimated. Among the four saline tolerant genotypes of mulberry **S-1635** suggested as a best genotypic variety because S-1635 has high nutritive value compared with the other 3 genotypes, also its growth and yield parameters is more convenient compared with other types. So, under the saline stress S-1635 gives good yield and also recommended as a best genotypic feed with high nutritive value for the Silkworm. The genotypes are recommended as follows based on their nutritive values, S1635, MR2, S36, and S13.

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KEYWORDS

Morus species, Mulberry, Silkworm, *Bombyx mori,* Saline tolerance

INTRODUCTION

Cultivation of silk worm for the production of silk fibre is called as "Sericulture". Silk is considered to be 'Queen of fibres' which is proteinecious in nature. Archeological and Bibliographical evidences shows that the sericulture was practiced in China about 2500BC [1]. In 12th century BC, it expanded outside China as Mulberry seed and silk worm egg were smuggled out. During 1920-30's world silk yield exceeds 60,000-70,000 tons, while in 1950-60's it decreased to 30,000 tons. After 1970, showed rapid development and has become the vocation of small agricultural families in popular developing countries like China, India, Vietnam, and Thailand. Current annual production of silk is about 4, 96,000 tons. India is 2nd largest producer of silk and the fact that nearly 6 million Indians are involved in sericulture[2].

Bombyx mori is an phytopagous insect which feeds only "Mulberry leaves". *Bombyx mori* can also be fed on mulberry leaves supplemented with *Spirullina furiformis.* The full genome of the silkworm was published in 2008 by the International Silkworm Genome Consortium [3].

Morus or Mulberry is a genus of 10–16 species of deciduous trees native to warm, temperate, and subtropical regions of Asia, Africa, Europe, and the Americas, with the majority of the species native to Asia. The closely related genus *Broussonetia* is also commonly known as mulberry, notably the Paper Mulberry, *Broussonetia papyrifera*. Mulberries are fast-growing when young, but soon become slow-growing and rarely exceed 10-15 metres (33-49 ft) tall. The leaves are alternately arranged, simple, often lobed, more often lobed on juvenile shoots than on mature trees, and serrated on the margin. A study to determine the nutritive value of mulberry (*Morus alba*) leaves in sheep diets was conducted by Kandylis *et al*[4].

Any kind of fluctuation in the environmental conditions is called as "Stress". Soil salinisation is a problem in the entire world and it has grown substantially causing loss in crop productivity. It has been estimated that about 954 million hectares of land around the world are already salinized and 4.5% of these lands are located in Brazil. Soil salinity is one of the most important agricultural problems in arid and semiarid climate conditions in different parts of the world[5]. Salinity is one of the limiting factors of crop yield in nearly one third of irrigated land. Among abiotic stresses, high salinity stress is the most severe environmental stress, which impairs crop production on at least 20% of irrigated land worldwide[6]. Sericulture productivity is more dependent on irrigation or partial irrigation since mulberry responds well to irrigation; as evidenced by intensive cultivation in Southern and Eastern plains of India, where nearly 40% of mulberry cultivation is under irrigation. Salinity influence growth in mulberry through reduction in root growth at lowers levels and at higher level it has greater effect through reduction of shoot growth. Above 30 mM of salinity drastic reduction in growth of both shoot and root are reported. Salinity also strongly decreases the protein, starch, and sucrose levels in leaves. Protocol for screening of large number of germplasm accession of saline tolerant lines in mulberry, through *in vitro* screening, using lower concentrations of NaCl at 0.1% and 0.2% level to know the effect on rooting has been suggested [7,8].

Salinity reduces plant growth due to osmotic and ionic effects on soil solutions. Short term

effects include reduction on growth by salt due to osmotic effects, which reduces cell expansion. Long term effects include excessive salt adsorption, which causes plants to suffer ionic stress, leading to premature leaf aging following a reduction in the available photosynthesis area to maintain growth[9].Soil salinity affects plant growth and development due to harmful ion effects and water stress caused by reduced Osmotic potential in the soil solutions[10].

MATERIALS AND METHODS

(i) Preparation leaf extract:

500mg of leaf sample from the genotypes S1635, S36, S13, and MR2 were collected separately and washed well to remove surface dusts. Then the leaf samples are grained well with distilled water using mortar and pestle, separately. Grained mixture of leaf was centrifuged at 10000 rpm for 10min, the supernatant was taken out and used as leaf extract, the concentration of leaf extract is 1mg/ml.

(ii) Estimation of total leaf protein by Lowry's method:

The total leaf protein was estimated using Lowry's method[11]. Pipetted out different aliquots of Standard protein (BSA) solutions into various test tubes (S1, S2, S3, S4, S5, for). The leaf samples extract of 1ml from different genotypes was added in test tubes named as T1- S1635, T2- S36, T3- S13, and T4- MR2**.** The content in the tubes were made upto 2ml with distilled water. To each tube added 2ml of Lowry's reagent mixed well and allowed to stand at room temperature for 10minutes.Then added 0.5ml of Folin's reagent and allowed to stand for 30min in dark till the colors develops. The color developed test tubes was read at 560nm.A standard graph was plotted by taking concentration of protein in the X-axis and absorbance in the Y-axis. From the graph, the amount of protein present in the test tubes was calculated. Blank was prepared by using distilled water.

(iii) Estimation of total soluble carbohydrate in leaf (Anthrone method)[12]:

Pipetted out different aliquots of Standard sugar (Glucose) solutions into various test tubes (S1, S2, S3, S4, S5). The leaf samples extract of 1ml from different genotypes was added in test tubes named as T1- S1635, T2- S36, T3- S13, T4- MR2.Blank was prepared by using distilled water. The volumes were made up to 3ml by using distilled water. Then the Anthrone solution of 5ml was added to all the tubes, and kept in boiling water bath for 15 minutes. After the color change occur in tubes, the tubes were taken out from bath and cooled by using running tap water. The color intensity developed was read at 600nm using UV Spectroscopy.

(iv) Estimation of ascorbic acid in leaf:

Ascorbic acid of leaf samples were estimated by titration method using DCPIP (2, 6-dichlorophenolindophenol) dye. Pipette out 5ml of the working standard solution into a 100ml conical flask. Add 10ml of 4% oxalic acid and titrate against the dye (V1ml). End point is the appearance of pink color which persists foe minutes. The amount of the dye consumed is equivalent to the amount of ascorbic acid. Extract the leaf sample (500mg) in 4% oxalic acid and make up to a known volume (100ml) and centrifuge. Pipette out 5ml of this supernatant, add 10ml of 4% oxalic acid and titrate against the dye (V2 ml). Amount of Ascorbic acid mg/g of sample was calculated by the following formula,

Ascorbic acid mg/g = (0.5mg/V1ml) × (V2/5ml) × (100ml/wt of Sample taken)×100

(v) Determination of Proline in plant leaf[13]:

Proline and free amino acid content which acts as a osmoprotectants are estimated by plotting the standard graph with known concentrations of L-proline and L-leucine separately. Pipetted out different aliquots from the working standard (L-Proline) to the test tubes of S1, S2, S3, S4, S5. Blank was prepared by using distilled water. Leaf sample tubes are prepared by 1ml of leaf extract using different genotypes. Volume of test tubes made up to 2ml in each tube with double distilled water. Then 2ml of Glacial acidic acid was added to the tubes. 2ml of acid nin-hydrin was then added to the tubes. The tubes were kept for incubation in boiling water bath for 1hour.Reaction was terminated by placing the tubes in ice-bath. Added 4ml of Toluene to each tube and stir well for 20-30 sec. A pinkish red colored toluene layer will be formed on top. Separated the layer in another tube carefully and warm to room temperature. Measured the pinkish red color at 520 nm. Use quartz cuvettes only. The color is stable for 1hour.The amount of proline present in the leaf sample of different genotypes was calculated by plotting standard graph.

(vi) Estimation of total free amino acid extraction of amino acids from the leaf:

0.5g of leaf sample was weighed and homogenized with 5ml of 80% ethanol. The homogenate was centrifuged at 15000 rpm for 15min. the residues was re-extracted with 5ml of 80% ethanol and centrifuged. The supernatants were pooled and used for quantitative estimation of total free amino acid. 1ml of nin-hydrin solution was added to 0.1ml of extracts in test tubes. The volume was made up to 2ml with distilled water. The tubes were heated in a boiling water bath for 20min. 5ml of diluents solvent was added and the contents were mixed well. After 15min, the absorbance of the purple color was added at 570nm.A standard curve was prepared by using L-leucine. Using the standard curve the amount of free amino acids present in the leaf samples was calculated. The free amino acids content was expressed in terms of mg/g f.w (fresh weight).

(vii) Estimation of total phenol content of leaf[14]:

Weigh exactly 0.5g of leaf samples of different genotypes and grind it with a pestle and mortar in 10-time volume of 80% ethanol. Centrifuge the homogenate at 10,000rpm for 20min. save the supernatant. Re-extract the residue with five times the volumes of 80% ethanol, centrifuge and pool the supernatants. Evaporate the supernatant to dryness. Pipette out aliquots (0.2ml to 1.0ml) into test tubes. Make up the volumes in each tube to 3ml with double distilled water. Add 0.5ml of folin-ciocalteau reagent. After 3min, add 2ml of 20% Na2 CO3, solution to each tubes. Mix thoroughly, place the tubes in a boiling water bath for exactly 1min, cool and measure the absorbance at 650nm against a reagent blank. Prepare a standard curve using different concentration of gallic acid. From the standard curve the concentration of phenols in the test samples were estimated.

(viii) Membrane permeability determination (EL: electrolytic leakage)[15]:

One gram of fresh sample (root/leaf segments) was collected from each genotype. Samples were washed three times with double distilled water to remove the surface – adhered electrolytes. Plant segments were divided equally and placed in two closed vials containing 20ml of double distilled water. One vial was incubated at 25^0c on a Rotary shaker for 3hours.The electrical conductivity of the solution (EC1) was determined with conductivity meter. The second vial was incubated/ autoclaved at 120^0C for 20 min. and electrical conductivity of the solution (EC2) was determined following a 25^0C equilibration.

EL was defined as **EL (%) = EC1 / EC2 × 100**
EC_1 – EC value of Leaf incubated at 25^0C for 3 hr
EC2 – EC value of Leaf autoclaved at 120^0C for 20 min

(ix)Measurements of chlorophylls & carotenoids[16]:

100mg of leaf sample from different genotypes were collected and homogenized in 20ml of 80% Acetone using mortar and pestle. The homogenate was centrifuged at 5000rpm for 5min and the supernatant is collected in a volumetric flask. The residue is re-extracted with 20ml of 80% Acetone and centrifuged. Supernatant is collected in the same volumetric flask and the final volume is made up to 100ml. Then the absorbance of the extract is taken at 663nm, 646nm, and 470nm.Amount of chlorophyll a, b, total chlorophyll and total carotenoids are calculated as follows,

Chlorophyll **a:** 12.25 × A (663nm) – 2.79 ×A (646nm) µg/ml.
Chlorophyll **b:** 21.50 × A (646nm) – 5.10 × A (663nm) µg/ml.
Chlorophyll **a+b:** 7.15×A (663nm) + 18.71 ×A (646nm) µg/ml.
TOTAL carotenoid : 1000 × A(470nm) – 1.82Chl a – 85.02Chl b / 198 µg/ml
Where A = absorbance

(X) Xylem SAP pH of leaf & Stem:

Xylem sap pH of leaf and stem was viewed by using the pH strip or paper.

The gel like liquid will come out while cutting a part of the leaf and stem. The pH of that sap material is viewed just by using the pH paper.

(xi) Estimation of chlorophyll stability index:

Chlorophyll stability index was (CSI) is determining as outlined by sivasubramaniawn (1992)[17]. Two grams of fresh green leaf is taken and divided into two lots of one gram each. One lot (control) is stored in room temperature (26***c**) and other lot put in empty test tube standing in boiling water bath for one hour. The total chlorophyll content of the two lots is measured as described earlier. CSI is calculated using the following formula:

$$\textbf{CSI (\%)} = \frac{\text{Total chlorophyll content (heated)}}{\text{Total chlorophyll content (control)}} \times 100$$

(xii) Growth and yield parameters:

YIELD AND YIELD CONTRIBUTING TRAITS: All the yield and yield contributing parameters were recorded at the time of harvest i.e., 70 days after imposing the treatments.

TOTAL SHOOTH LENGTH: The height of all the primary shoots of each plant was measured in centimeter from the base of the plant to the tip using a meter scale. The length of all the branches of a plant was added to obtain the total shoot length.

LEAF YIELD (FRESH WEIGHT): Leaves of each plant were harvested separately and the weight recorded as fresh weight of leaves in grams.

STEM WEIGHT (FRESH WEIGHT): After the leaves were harvested, the stems of each plant were pruned separately and the weight recorded as fresh weight in grams.

BIOLOGICAL YIELD (FRESH WEIGHT): The leaf weight and stem weight obtained from each plant were added to obtain the biological yield and the weight was recorded in grams.

ROOT WEIGHT (FRESH WEIGHT): Roots of each plant were taken separately after washing and the weight recorded as fresh weight of roots in grams.

RESULTS

The study to determine the nutritive value of mulberry (*Morus sps.,)* leaves, under the saline stress condition was conducted. Here four major saline tolerant genotypes of mulberry (S1635, S36, S13, and MR2) were taken for the test, and their nutritional values were estimated and recorded in the Table 1. The graphical comparison was described in the chart (fig.1-6) for easy understanding.

DISCUSSION

From the results obtained through the different biochemical tests, among the four saline tolerant genotypes of mulberry S-1635 suggested as a best genotypic variety. Because S-1635 has high nutritive value compared with the other 3 genotypes, also its growth and yield parameters is more convenient compared with other types. So, under the saline stress S-1635 gives good yield and also recommended as a best genotypic feed with high nutritive value for the Silkworm.

Other recommended genotypes have less quality compared with S-1635. The genotypes are recommended as follows based on their nutritive values, S1635, MR2, S36, and S13.

Fig.1
Comparison of carbohydrate and protein levels

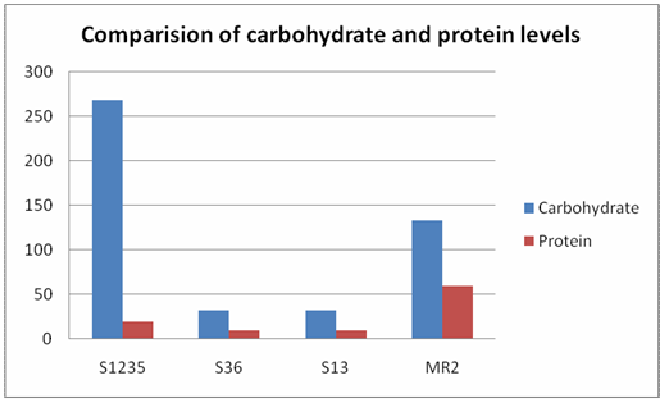


Fig.2
Comparative chart of ascorbic acid, proline, aminoacid, phenol, chlorophyll a,b, total chlorophyll and total carotenoid

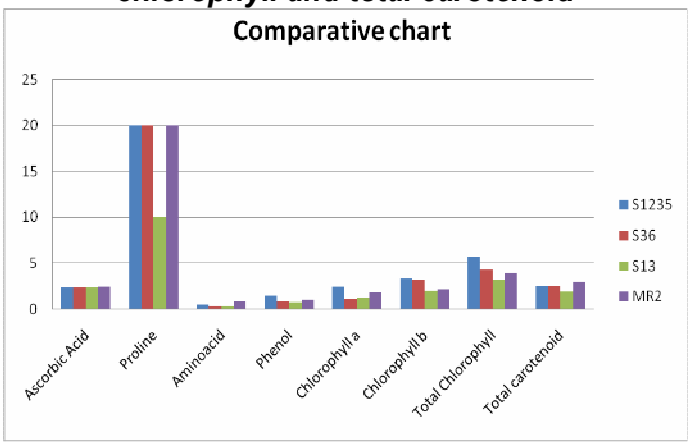


Table 1
Nutritive value of mulberry under the saline stress condition

ESTIMATED PARMETERS	S 1635	S 36	S 13	MR 2
Estimation of total protein by Lowry's method (mg/g F.Wt)				
Protein	20	10	10	60
Estimation of total soluble carbohydrate(mg/g F.Wt)				
Carbohydrates	267.5	32.55	32.55	132.5
Estimation of ascorbic acid content (mg/g F.Wt)				
Ascorbic acid	2.333	2.233	2.266	2.366
Estimation of total proline in leaf (mg/g F.Wt)				
Proline	20	20	10	20
Estimation of free amino acid content (mg / g F. Wt)				
Free amino acid	0.4	0.3	0.3	0.8
Estimation of total phenolic content (mg/g F. Wt)				
Phenols	1.4	0.8	0.7	1.0
Measurement of chlorophyll and Carotenoids				
Chlorophyll a	2.354	1.093	1.257	1.815
Chlorophyll b	3.291	3.207	1.917	2.092
Total chlorophyll	5.645	4.259	3.179	3.912
Total carotenoids	2.454	2.451	1.842	2.924
Chlorophyll ratio	1:1.4	1:2.9	1:1.5	1:1.5
Chlorophyll-carotenoids ratio	2.3:1	1.7:1	1.7:1	1.3:1
Measurement of Chlorophyll stability index (%)				
Chlorophyll stability index	86.74%	92.02%	82.66%	84.93%
Measurement of Electrolytic leakage of leaf (%)				
Electrolytic leakage of leaf	43.839%	32.061%	42.804%	30.566%
Xylem sap pH of leaf & stem				
pH OF Leaf	6.2 ± 0.2	6.0 ± 0.3	6.6 ± 0.2	6.3 ± 0.1
pH OF Stem	6.1 ± 0.1	6.2 ± 0.2	5.8 ± 0.1	5.9 ± 0.2
Growth & yield parameters (70^{th} day)				
Total shoot length(cm)	18	10	9	14
Leaf Number	115	70	65	98
Leaf weight (Kg)	4.4	2.3	2.1	3.2
Stem weight (Kg)	6.2	2.9	3.0	4.0
Biological Yield (Kg)	10.6	5.2	5.0	7.2
Root weight (Kg)	0.65	0.28	0.25	0.48

Fig.3
Chlorophyll stability index and Electrolytic leakage of leaf

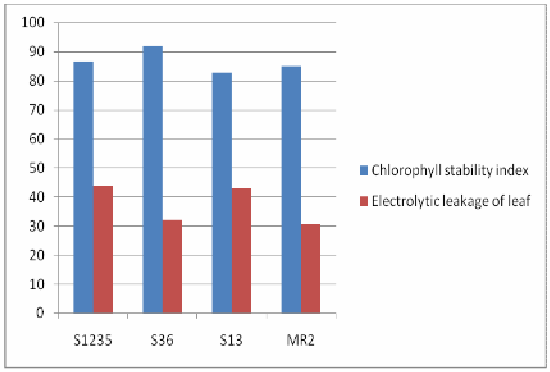


Fig.4
Growth and yield parameters- shoot length

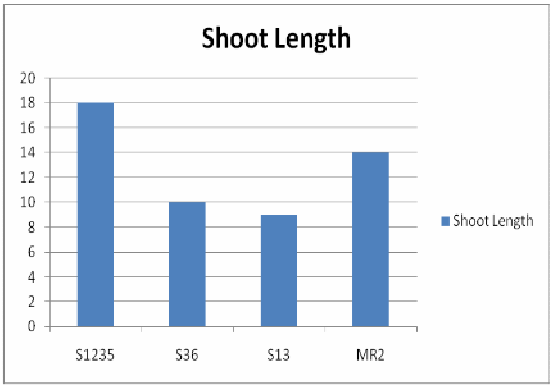


Fig.5
Growth and yield parameters- Shoot numbers

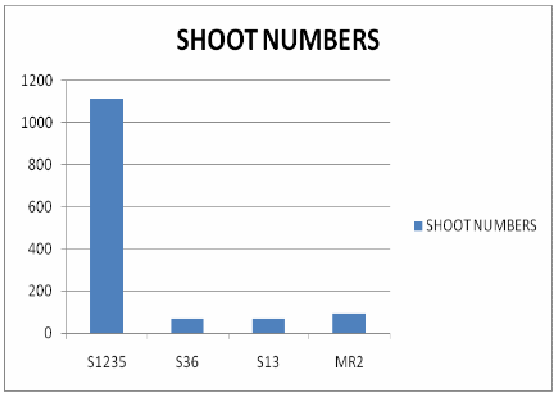
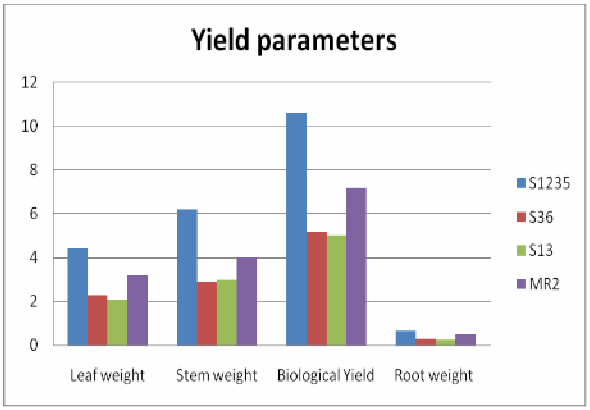


Fig.6
Yield parameters



CONCLUSION

The above result and discussion confers S-1635 has high nutritive value compared with the other 3 genotypes also its growth and yield parameter is more convenient compared with other types. So, under the saline stress S-1635 gives good yield and also recommended as a best genotypic feed with high nutritive value for the Silkworm.

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