International Journal of Zoology and Applied Biosciences **ISSN: 2455-9571**
Volume 9, Issue 5, pp: 8-14, 2024
https://doi.org/10.55126/ijzab.2024.v09.i05.002 **http://www.ijzab.com**

Research Article

IDENTIFICATION OF BIVOLTINE BREEDS TOLERANT TO HIGH TEMPERATURE AND HUMIDITY USING MARKER ASSISTED SELECTION IN SILKWORM *BOMBYX MORI*

Raviraj VS, *Md. Akib Hussain, Anu Sonowal and Y. Surjalata Devi

Central Sericultural Research and Training Institute, Berhampore-742101, West Bengal, India

Article History: Received 15th May 2024; Accepted 4th August 2024; Published 1st September 2024

ABSTRACT

The growth of bivoltine silk production in eastern and north-eastern india lags behind drastically in comparison to southern states due to non-availability of suitable bivoltine hybrids. The major constraints in bivoltine popularization are adverse climatic conditions. to overcome this limitation and to continuously rear bivoltine hybrids in these harsh climatic conditions throughout the year, the present proposal aims to identify robust and abiotic stress tolerant bivoltine silkworm breeds through integrating conventional breeding (directional selection) for improved productivity and advanced genome technologies such as MAS (marker assisted selection), gene specific markers for high humidity and thermo-tolerant DNA markers (Pyx3, Pyx4, S0803 and S0816) for tolerance to high temperature and high humidity and other abiotic factors. These robust hybrids could be reared throughout the year and would provide opportunities for further popularizing and improving bivoltine raw silk production in the eastern and north-eastern region. the present investigation identified wb1hh bivoltine breed to be having highest survival rate and better shell ratio of 18.81% in abiotic stress condition. MAS selection identified DNA markers in all breeds and among them WB1HH showed all four markers, confirming the genetic ability of the breed for tolerance to high temperature and humidity.

Keywords: Abiotic stress, Bivoltine silkworm, DNA markers, Marker assisted selection.

INTRODUCTION

Mulberry silkworm is one of the most important domesticated insects, which produces luxuriant silk thread in the form of cocoon by consuming mulberry leaves during larval period. The growth and development of silkworm is greatly influenced by environmental conditions. The biological as well as cocoon-related characters are influenced by ambient temperature, rearing seasons, quality mulberry leaf, and genetic constitution of silkworm strains. The success of the silk industry depends on several factors of which the impacts of environmental factors such as biotic and abiotic factors are of vital importance. Among the abiotic factors, temperature and humidity plays a major role on growth and productivity in silkworm, as the poikilothermic insect (Benchamin and Jolly, 1986). It is known that the late age silkworm prefers relatively lower temperature than young age (Krishnaswami, 1994) and fluctuation of temperature and humidity during different stages of larval development was found to be favourable for growth and development of larvae than constant temperature. There is ample literature showing that good quality cocoons are produced within a temperature range of 22-27°C and above this level makes coccon quality worse (Krishnaswami, 1994). However, multivoltine races reared in tropical countries are known to tolerate slightly higher temperature. It is also true with crossbreeds which have been evolved specially for tropical climate.

Under tropical condition, unlike multivoltines, bivoltines are more vulnerable to various stresses like hot and humid climatic conditions of tropics, poor leaf quality, and improper management of silkworm crop during summer that is not conducive for bivoltine rearing for technologically and economically poor farmers of India. In order to introduce bivoltine races in a tropical climatic region like eastern India, it is necessary to have stability in

*Corresponding Author: Md. Akib Hussain, Central Sericultural Research and Training Institute, Berhampore -742101, West Bengal, India, Email: hussainakib0310@gmail.com, Mobile: +91 9365049783.

cocoon crop under high temperature and high humidity environment. In order to have stable cocoon crop this project was designed to screen the better performing silkworm breed in eastern India in unfavourable seasons with both survivability and productivity in consideration. The present investigation has resulted in selecting better adapting silkworm breed for high temperature and high humidity conditions after subjecting 11 silkworm breeds under high temperature and high humidity stress in rearing season from June-July.

MATERIALS AND METHODS

Silkworm rearing and humidity treatment

Present investigation was carried out at Central Sericultural Research & training institute, Berhempore to determine the effect of change in temperature and humidity on the survival of Bombyx mori. L larvae and to identify bivoltine breeds tolerant to high temperature and humidity which will enhance silk production. Total 6 silkworm breeds had been studied for this experiment. Of the 6 silkworm breeds, five are dumbbell in shape namely (WB1HH, BB4HH, SK7HH, N5, CSR4) and one breeds are oval in nature (CSR2). Silkworm rearing was conducted following the standard method under natural conditions and also at recommended temperature and humidity conditions for control batches at 25 ± 2ºC and 65 ± 5%RH. A batch of 100 worms for each breeding line was exposed for high temperature and humidity treatment to assess their survivability under harsh condition at 36±1ºC and 85±5%RH. Only late age larvae were exposed for high temperature and humidity treatment after completion of Day 2 and stress treatment was continued till Day 5 for 6 hours (10:00 to 16:00hrs) in environmental chamber giving consistent and maintaining the room temperature for the abiotic stress treatment.

DNA Isolation and Marker Analysis

For genomic DNA isolation moths from the investigation group was collected and homogenized the moth sample with 1ml of 2PK buffer (Tris-HCl 24.20 gm, EDTA 9.216 gm and NaCl 17.52 gm in 1000 ml of distilled water) containing 2µl of Proteinase K (50µg/ml) and incubate at 65 ºC for 30 minutes. 1.3µl of RNaseA was added and again incubated for 30 minutes in 37 ºC for 30 minutes. After digestion with Proteinase K, a phenol-chloroform-isoamylalcohol extraction was performed and the DNA was precipitated out with isopropanol. Purified DNA was dissolved in 0.1X TE buffer (pH 8.0). For marker assisted selection S0803, S0816, pyx3 and Pyx4 markers were selected as they were associated with high temperature tolerance. Of the above mentioned markers S0803 and S0816 are SSR markers and Pyx3 and Pyx4 are SNP markers. Primers were designed for the markers and procured for the analysis from the gDNA isolated from the moths using conventional PCR method. The PCR were performed for all the DNA markers (S0803, S0816, Pyx3 and Pyx4) under the study by preparing master mix of the following the reaction mixture and programme set up using Corbett PCR machine. A total volume of 10 µl was prepared for each reaction and then placed inside the PCR machine. For DNA markers, PCR conditions were as follows: 1) 95°C for 3 minutes, 2) 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds, and 3) a final extension step of 5 minutes at 72°C. The amplified PCR products were confirmed using in 1.5% agarose gel at a constant Voltage of 80V for 2 hours and visualize under Gel documentation system. The gel images are recorded for identification marker bands.

Rearing parameters

Various quantitative traits such as single cocoon weight, single shell weight and cocoon shell percentage, ERR (by wt & no) were calculated. Hatched out larvae from the DFLs (disease free laying), collected into pre-disinfect rearing trays and were daily feed three times on fresh mulberry leaves except during moulting. While cleaning the unconsumed leaves, two times during 1st and 2nd instar and once every day after 2nd moult, the larvae were separated pre disinfected rearing trays. The larvae under moult, however, were not disturbed. During the process of rearing after 3rd instars, 300 no of larvae in each breeds were to be taken and kept it to high temperature and high humidity stress and observed survivability rate of each breeds daily. Assessment was carried out on the subsequent day. The survival rate was calculated as the number of live pupae to the number of larvae treated. And noted which variety would be survived. The mature larvae on the 6th/7th day of 5th instar were picked and mounted on to the mountages. The cocoons were harvested on 6th day and the floss of the cocoons was removed. During rearing, data on the economically important traits like fecundity, hatching percentage, yield by weight are recorded. In addition to rearing parameters, morphological characters like larval marking, cocoon colour and cocoon shape were observed for the present study. And also observed the defective cocoons which was greatly depends on production of silk. It has been established that cocoon quality contributes to the tune of about 80 per cent of the raw silk quality. Cocoon quality is governed by several parameters; each of them has importance at certain level. Some of the important quality parameters usually considered is shell percentage, defective cocoon percentage, average filament length, average non-broken filament length, denier, and reelability percentage. Of these, shell percentage and defective cocoon percentage have been identified as the most significant ones especially because these are relatively easy to determine. lt has been established that each of the quality parameters has its own relative significance on the reeling efficiency and raw silk quality.

RESULTS AND DISCUSSION

Rearing performance was carried out using important parameters for the study such as ERR by number, ERR by weight and shell ratio. ERR by number was highest in

N5HH and WB1HH showing better survival rate and had the highest effective rate of rearing in the control batches and CSR4 had the highest shell ratio but the survival was lower of CSR4 in control conditions. In the abiotic stress treatment batch where the bivoltine breeds were exposed to high temperature and humidity WB1HH showed the highest ERR by number showing its effective survival under stress condition while CSR2 and CSR4 were the least performing ones. On contrary CSR4 showed the highest shell ratio among all the breeds in abiotic stress treatment batch. The ERR, single shell weight and cocoon weight was calculated for all the batches in control group and treatment group and depicted in table 1. The calculations are mentioned in the methodology.

Table 1. Rearing performance of the bivoltine breeds under the present investigation.

Breeds	C/T	Basic Larva (By No.)	GC	Actual Yield (By No.)	Actual Yield (By Wt.)	ERR (By No.)	ERR (By Wt.)	SCW (gm)	SSW (gm)	Shell %
WB1 HH	C	300	258	288	354	9600	12.29	1.516	0.270	18.81
	T	100	78	80	126	9091	15.75	1.596	0.274	17.16
BB4 HH	C	300	217	239	253	7967	10.59	1.48	0.260	17.57
	T	100	66	66	90	7500	13.64	1.297	0.226	17.47
SK7 HH	C	300	175	213	193	7100	9.06	1.441	0.239	17.61
	T	100	48	48	66	6705	13.75	1.160	0.194	16.72
N5 HH	C	300	255	281	286	9367	10.18	1.438	0.269	18.49
	T	100	78	78	107	8864	13.72	1.497	0.269	17.97
CSR2	C	300	185	250	215	7300	10.31	1.349	0.264	19.57
	T	100	25	25	32	2841	12.80	1.207	0.198	16.40
CSR4	C	300	221	271	265	9600	10.08	1.279	0.306	23.92
	T	100	59	59	75	5455	12.71	1.137	0.238	20.93

C- Control, T- Treatment, GC- Good Cocoon, ERR- Effective Rate of Rearing, SCW- Single Cocoon Weight, SSW- Single Shell Weight

Survival Rate (Pupation %): Survival rate was assessed by calculating the healthy pupae in the cocoons harvested in the control batches and experimental rearing batches. Unhealthy pupae or the dead pupae were counted as dead even though they formed cocoons. WB1HH showed the highest survival rate of 90% under stress condition followed by N5HH and BB4HH with 88% and 84% respectively.

Table 2. Survival data of control group and abiotic stress treatment group under the present investigation.

Breeds	Batches	Basic larvae (By Number)	Survival Rate (%)
WB1HH	Control	300	96
WB1HH	Treatment	100	90
BB4HH	Control	300	84
BB4HH	Treatment	100	75
SK7 HH	Control	300	71
SK7 HH	Treatment	100	67
N5 HH	Control	300	93
N5 HH	Treatment	100	88
CSR2	Control	300	61
CSR2	Treatment	100	28
CSR4	Control	300	73
CSR4	Treatment	100	54

DNA Marker analysis: DNA marker analysis was conducted utilizing four markers associated with high temperature and High Humidity (S0803, S0816, Pyx3, and Pyx4). The molecular markers were assessed for Pyx 3 and Pyx 4 was based on their presence or absence SNPs in each breeds. Among the investigating breeds all the breeds except SK7HH, CSR2, and CSR4 did not had the presence of these markers while all the other breeds had presence of the marker. Since WB1HH showed highest survival rate we suggest taking WB1HH forward for next generation of breeding experiments. S0803 and S0816 SSR markers were also assessed in all the breeds and presence of homozygosis markers confirm their presence of tolerance in the given group. Although all the breeds had these markers; correlation with pupation rate was an important criterion for the study.

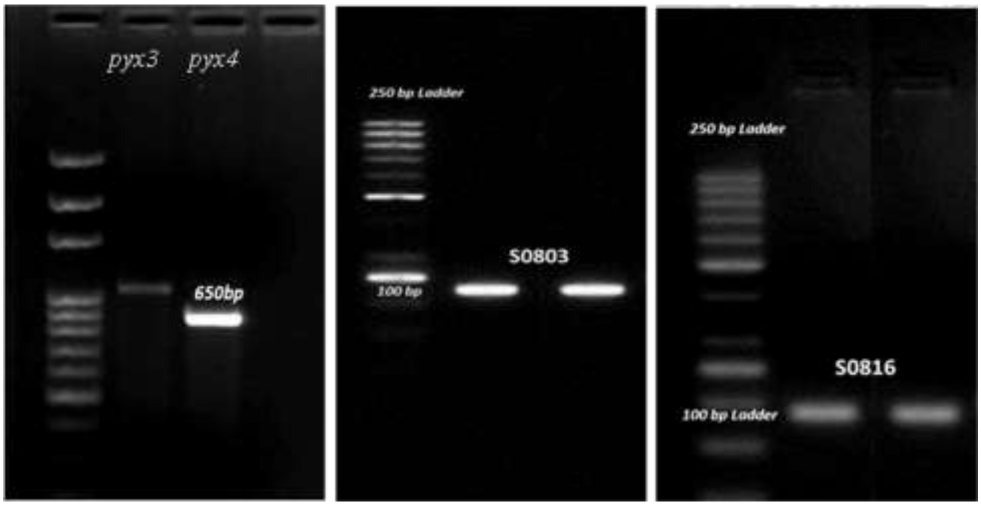

Figure 1 : Image represnting the DNA Markers amplified in the bivoltine breeds under the present investigation.

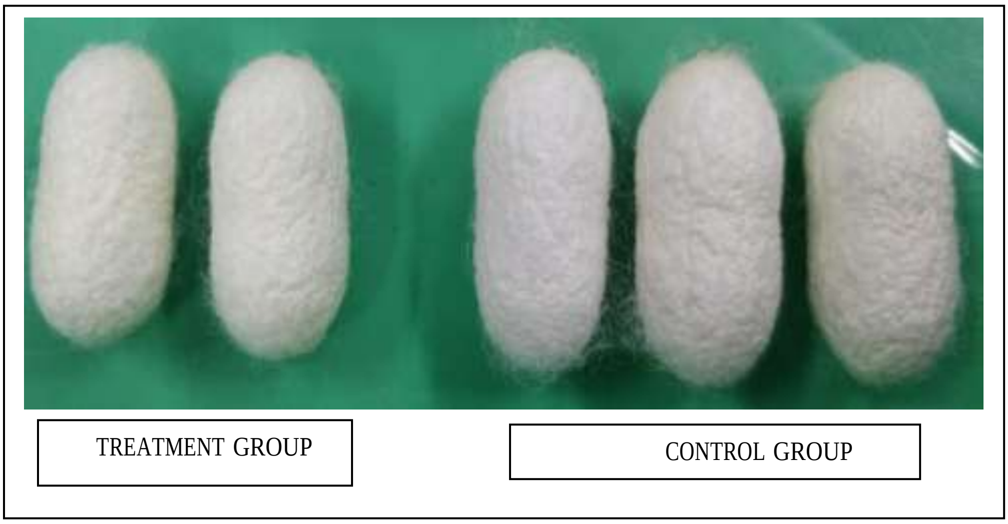

Figure 2. Images of cocoons in comparison with control group and treatment group.

Effect of high temperature and humidity on cocoon weight and size: Effects of high temperature and humidity was observed in the cocoon size has the treatment group was reduced in size and the cocoon and shell weight had decreased from the control as represented in rearing performance table. Considerable amount of shell ratio was lowered in the treatment group of the experimental batches. The breeding of silkworm since long has been aimed towards evolving of superior and hardy breeds either by means of selection alone or by combining out-crossing or backcrossing with selection in the subsequent generations. The final aim of the breeder is primarily to evolve a breed which can give rise to stabilized crops and secondly to improve both quantity and quality of silk (Tazima, 1984).

The breeding of silkworm races probably dates back to the beginning of the history of silkworm rearing, but it has made great progress rather recently (Hirobe, 1968). Sericulturally, advanced countries like Japan has achieved remarkable progress by executing systematic breeding plans for the development of productive races. In silkworms, studies carried out for various characters have shown that the characters could be changed to suit the breeders choice, since selection for one trait has correlation with genetic change of other characters. The selection of breeding resource material helps the breeder to successfully amalgamate desired traits. Appropriate experimental design, selection methods employed in fixing the major traits contributing to the improved cocoon yield leads to the success of any breeding programme. Besides, understanding the genetic diversity of parental strains to be utilized in the breeding programme by their systematic evaluation, critical assessment of their quantitative nature which is greatly influenced by the environmental factors such as temperature, light, relative humidity, nutrition and rearing techniques which paves the way for the breeder for effective utilization (Kogure, 1933; Legay, 1958; Ueda and Lizuka, 1962; Suzuki et al.,1962; Yokoyama,1963; Ito and Arai, 1967; Horie *et al*., 1967; Naseema Begum et al., 2001 and Sudhakar Rao *et al*., 2001).

According to Allard and Bradshaw, 1964, performance of the strain itself in a given environment indicates its superiority. While evaluation, emphasis was given on the phenotypic expression of traits of economic importance under different temperature conditions. However, as the objective of the study was for greater viability and high productivity merits, equal importance was given on these two traits while selection of parents. The significant variations observed in the phenotypic manifestation for the traits analyzed can be attributed to the genetic constitution of the breeds and their degree of expression to which they are exposed during their rearing. Such variations in the manifestation of phenotypic traits of the breeds studied can be ascribed to the influence of environmental conditions. Variable gene frequencies at different loci make them to respond differently. The results are in line with the findings of (Watanabe, 1928; Hassanein and Sharawy, 1962; Krishnaswami and Narasimhanna, 1974; Ueda et al., 1975; Rajanna, 1989; Raju, 1990; Maribashetty, 1991;Kalpana, 1992; Nirmal Kumar,1995; Basavaraja, 1996 and Sudhakar Rao et al., 2001). It is important to measure the phenotypic expression of the major contributing traits of economic importance in the silkworm strains under diversified environmental conditions to understand the genetic endowment pertaining to adaptability and productivity. The balancing of desirable traits during the course of the breeding for varied climatic conditions is a challenging task for the breeder. The choice of parental material is critical and difficult to evaluate all the available silkworm breeds,. However, a few strains of known genetic background, pedigree and specific traits desirable for the new breeding programme have to be taken into consideration while evaluating the breeding material. It is also equally important to understand the traits related to productivity and viability. All the breeds selected for the evaluation are having one or more desirable traits as per the objectives of the present study. In the present study, which envisages to evaluate bivoltine breeds to identify more resistant bivoltines that can give rise to stable cocoon crops with better viability, even though productivity is low compared to the existing productive bivoltine breeds that are currently used in the field. In silkworms, the correlation for some characters is positive and for some it is negative (Gamo and Ichiba, 1971 and Gamo, 1976). Such a negative correlation is observed for the traits productivity and viability and hence the attempt made was to increase the viability of the developed breeds. Moreover as suggested by Lekuthai and Butrachand (1974) and Strunnikov (1986), the selection parameters were primarily aimed at improving the viability character such as yield by number without sacrificing much of the productivity traits like cocoon weight, cocoon shell weight and yield by weight. In addition, during later generations of inbreeding, selection was applied to select desired genotypes to improve the traits of commercial importance like viability and productivity as suggested by Mano (1993 and 1994) to improve the yield of bivoltines.

CONCLUSION

Eastern and North Eastern India is generally characterized by luxuriant growth of mulberry for its highly fertile soil and rainfall. But, rearing of productive silkworm breeds and hybrids are restricted due to highly variable climatic situation, which causes poor larval growth, moulting disorder and severe mortality of silkworm caused by diseases and ultimately leading to low cocoon yield. High temperature coupled with high humidity act as the major limiting factor for rearing silkworm particularly during the seasons falling between May and September when huge leaf biomass is available. Low temperature with low humidity during December and January severely affects the performance of P1 rearing. Besides, due to low production of mulberry leaf in low temperature regions, which affects the growth of silkworm resulting in poor performance of bivoltine rearing. Though, February- March season is suitable for raising bivoltine as a P1 but subsequent commercial rearing for bivoltine high humidity tolerant breeds which was unpredictable due to prevalence of high temperature and high humidity during late age rearing. In West Bengal the bivoltine parent rearing was found to be difficult during unfavourable season due to the prevalence of high temperature and high humidity. Further bivoltine seed crop stabilization, which is bottleneck for the development of sericulture in the region, can also be solved by the development of sustainable bivoltine breeds suitable to the West Bengal conditions. Hence, developing breeds or hybrids by utilizing the molecular approach of marker assisted selection for thermo tolerance may change the status of bivoltine sericulture in this region of India. These approaches are high robust and selection is rigorous throught out the breeding seasons. Developing these tolerant breeds will not only strengthen the economy of the farmers but also helps in enhancing the silk productivity globally.

ACKNOWLEDGMENT

The authors extend their gratitude to Dr. A.R. Pradeep, Head of the Biotechnology division at the Central Sericultural Research and Training Institute, as well as to all the technical staff for their invaluable guidance and assistance throughout the study period.

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