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Biotechnological importance of cocoon refrigeration on the pupal performance of multivoltine mulberry silkworm (*Bombyx mori Linn*).

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ABSTRACT

Temperature and humidity are the key environmental factors that play a major role in the physiological behavior of the insects. Being a poikilothermic organism temperature decides the fate of the development in silkworm. The insects will get acclimatized to the low temperature. The aim of the present study is to estimate the effect of cocoon refrigeration on the pupal performance i.e., pupal duration, pupal weight and survival of pupae. For the experiment cocoons were consigned to low temperature at 5°C at 0 ,2 ,4 , 6 ,8 days of pre-refrigeration and refrigerated for 0 ,5 ,10 ,15 ,20 ,25 ,30 days. A control set was always maintained with each batch. The maximum pupal weight (0.9074±0.023 g) was noticed in case of 8 day pre-refrigeration-0 day refrigeration and survival of pupae (85.33±1.54 %) was noticed in case of 6 day pre-refrigeration-0 day of refrigeration. While the minimum pupal weight (0.6226±0.008 g) and survival of pupae (55.10±1.776 %) was noticed in 6 day pre-refrigeration-20 day refrigeration of cocoon. The minimum pupal duration (7.89±0.35 days) was recorded in case of 0 day pre-refrigeration-0 day of refrigeration of cocoons. Thus it may solve the problem of sericulture industry regarding the development of a suitable long term seed cocoon preservation method for skipping safe post ponement of the emergence of moth.

Keywords: Refrigeration, Pre-refrigeration, Pupal duration, Pupal weight, Survival of pupae.

INTRODUCTION

The silkworm is economically important insects. India is the second largest producer of raw silk after china and the biggest consumer of raw silk and silk fabrics. In India, sericulture is not only a tradition but also a living culture. It provides employment at various levels i.e., host plant cultivation, silkworm rearing, reeling, spinning and weaving and have much impact on the improvement of rural economy. The ultimate aim of sericulture industry is the production of quality seed cocoons i.e. raw silk as per demand. In order to increase production of quality raw silk several efforts have been made to study the effect of ecological factors (Upadhyay et al., 2004), relative humidity (Upadhyay ad Mishra, 2002), refrigeration of eggs (Pandey and Upadhyay, 2000) and cocoon (Upadhyay et al., 2006 and Upadhyay et al., 2009) magnetization of eggs (Upadhyay and Tripathi, 2006) cocoon (Upadhyay and Prasad, 2010 a &b), larval performance (Prasad and Upadhyay, 2011). Phytoecdysteroid influenced pupal performances (Upadhyay and Pandey, 2012), *Aloe vera* essential oil (Tiwari et al., 2014) on the larval and pual performance of *B. mori*. The growth and development of silkworms is greatly influenced by environmental condition in which the most important are the atmospheric temperature and humidity prevailing at the time of rearing (Benchamin and Jolly 1986).Temperature plays a vital role on the growth of the silkworms. As silkworms are cold blooded animals, temperature will have a direct effect on various physiological activities. The temperature and growth of silkworms are directly proportional to each other. Wide fluctuation of temperature is harmful to the developmental of silkworm. A few workers (Vishwewara Gowda et al., 1987; Bheemanaa et al., 1989; Janarthanan et al., 1994; Khan et al, 1997; Pandey & Upadhyay 2001) studied the effect of refrigeration of silkworm eggs on the duration, weight and survival of pupae. The variations in the environmental conditions during the last decade emphasize the need of management of the temperature for sustainable production. The present study is an attempt to study the effect of refrigeration of cocoon on the pupal performance.

MATERIALS AND METHODS

Seed Cocoon: The seed cocoon (pupa enclosed in silken case) of multivoltine mulberry silkworm, *Bombyx mori nistari*, a native of west Bengal in India, was taken in the present study. The seed cocoon (pupa enclosed in silken case), obtained from the silkworm grainage Behraich, Directorate of Sericulture Uttar Pradesh, and were maintained in plywood trays (23×20×5 cm) under the ideal rearing condition (Krishnaswami et al., 1973) in the silkworm laboratory, Deen Dayal Upadhyaya Gorakhpur University, Gorakhpur. The temperature, relative humidity and photoperiod were maintained at 26±1°C, 80±5% RH and 12±1 hours light a day respectively till the emergence of moth from the seed cocoon. The moths emerged generally in the morning at around 4 am. The trays, in which seed cocoon were kept, were suddenly illuminated by light in the morning at 4O'clock on 9th and 10th day of spinning.

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The newly emerged moth, from seed cocoons, were quickly picked up and kept sex-wise in separate trays to avoid copulation. The male moths were smaller and more active than the female moths. The whole grainage operation was performed as per description given by Krishnaswami et al. (1973) and Jolly (1983).

Rearing of larvae: After two consecutive days of hatching, the silkworm larvae were collected with the help of bird's feather and reared to maintain a stock culture in the silkworm laboratory at 26±1°C, 80±5% RH and 12±1 hour light a day. For feeding, small pieces of fresh and clean leaves of *Morus alba* were given to the larvae and care was taken that food always remained in excess in the rearing trays. After completion of fifth instar, the ripe worms ceased feeding and ready for spinning. Now small montages were provided to the ripe worm. The ripe worms soon begin the mounting which was completed within three days. Thus sufficient number of cocoons was obtained from the silkworm larvae reared in the laboratory. These cocoons were taken for the purpose of experiments.

Experimental designing: The seed cocoons were obtained from the silkworm grainage and were maintained in the laboratory. The moths emerged were allowed to mate .After mating the female moths were allowed for egg laying. The eggs laid were transferred to the BOD incubator for hatching. The whole grainage operation was performed as per description given by Krishnaswami et al. (1973b) and Jolly (1983) .After hatching the larvae were collected and reared to maintain a stock culture in the laboratory. After completion of fifth instar, the moutages were provided to the ripe worms for the formation of cocoon. Thus sufficient number of cocoons was obtained which were used for further experiments to the refrigeration of cocoon.

Refrigeration of Cocoon: The cocoons obtained from the laboratory were refrigerated for different duration at varying conditions of pre-refrigerated periods of cocoons .The zero day (0 day) refrigeration of cocoons refers to be the control (no refrigeration of cocoon) .The 'pre-refrigeration' period refers to be the duration between the competition of the cocoon formation and beginning time of the refrigeration of experimental cocoon. During the pre refrigeration period, the cocoons were kept in BOD incubator maintained at optimum condition of temperature, relative humidity and photoperiod at 26±1° C, 80±5% RH and 12 hours dim light a day respectively.

For the refrigeration of cocoon 630 cocoons were consigned to low temperature at 5°C at 0 day pre-refrigeration period (one of the pre-refrigeration conditions i.e. control). The cocoons were refrigerated for 0, 5, 10, 15, 20, 25 and 30 days. For this purpose a group of 90 cocoons (30 cocoons in each of the three batches.) were released at once without any refrigeration (0 day pre refrigeration and 0 day of refrigeration) of cocoon which was taken as control. Further the rest of 540 cocoons were consigned at 5°C inside the refrigerator. After this a second group of 90 cocoons (three batches of 30 cocoons in each batch) were cold treated for 5 day refrigeration and were released from refrigeration accordingly in the groups of cocoons as in previous cases, after 10, 15, 20, 25 and 30 days of transferred chronically to BOD incubator maintained at optimum conditions of rearing. Three replicates of each experiment were made.

Like the above experimental designing at 0 day of pre refrigeration period, similar series of experiments were performed for the refrigeration of cocoons at 2, 4, 6 and 8 day of pre refrigeration periods. The moth emerged commonly in the morning at around 4:00 AM. The newly emerged moths were kept sex wise in separate trays to avoid copulation within the same group. Further, three batches each containing 5 good males and 5 good females were made and they were allowed to mate. After 4 hours of mating, the paired moths were decoupled manually. Further the gravid females were allowed to lay eggs on sheet of paper. The egg laying moths were covered by open plastic cellules to prevent the intermixing of egg masses deposited by different female moths. After 24 hours of egg laying, the female moths were individually examined for their disease freeness. The disease free laying (DFLs) thus prepared, were washed with 2 % formalin for 15 minute .To increase the adhesiveness of eggs on cards and surface disinfections. Thereafter the egg sheets, with eggs laid on were thoroughly washed with running water to remove formalin the eggs were dried in BOD incubators maintained in the laboratory. After two consecutive days of hatching, the silkworm larvae were collected with the help of brush and reared to maintain a stock culture in the silkworm laboratory at 26±1°C, 80±5% RH and 12±1 hours light a day.

Pupal weight: For determination of pupal weight 30 pupae (three batches of 10 pupae in each batch) were recorded. Three replicates of each experiment were made.

Pupal duration: For determining the pupal duration the time required from the 3rd day of spinning (formation of pupae) to the emergence of moth was considered. For this purpose, 75 cocoons along with pupae (three batches of 25 cocoons in each batch) were taken for observation.

Survival of pupae: For determining the survival of pupae 75 pupae (three batches of 25 normal pupae in each batch) were taken under the observation. The number of moth emerged as moth was counted for the calculation of the survival of pupae as following:

$$\text{Survival of pupae} = \frac{\text{No of moths emerged}}{\text{No of pupae taken for observation}} \times 100$$

RESULTS

Pupal Weight: The data given in table 1 clearly indicates that the duration of refrigeration period and pre-refrigeration of cocoon considerably influenced the pupal weight of silkworm. With the increasing duration of the refrigeration of cocoon, the pupal weight decreased from 0.868± 0.039 to 0.650 ± 0.10 g, 0.872±0.0419 to 0.640±0.009 g, 0.878±0.012 to 0.630± 0.012 g, 0.88±0.02 to 0.622±0.008 g and 0.907±0.02 to 0.661± 0.008 g respectively at 0, 2, 4, 6 and 8 days pre-refrigeration of cocoon. The trend of decline in the pupal weight due to increasing duration of refrigeration is almost of similar

fashion. The maximum pupal weight was noticed to be 0.907±0.023g, obtained from the untreated cocoon while the minimum pupal weight 0.622±0.008 g was obtained from 6 days pre-refrigerated cocoon, refrigerated for 20 days. Two way ANOVA indicates that both, the pre-refrigeration period and duration of refrigeration have significant (P<0.01) influence on the pupal weight of *B. Mori.*

Pupal duration: The data presented in table 2 clearly indicates that the duration of refrigeration period and pre-refrigeration of cocoon considerably influenced the pupal duration of silkworm. With the increasing duration of the refrigeration of cocoon, the pupal duration increased from 7.89 ±0.35 to 10.01 ±0.36, 8.19 ±0.09 to 11.33±0.046 ,8.22±0.112 to 11.31±0.048 ,8.47±0.266 to 10.92± 0.3 and 8.77±0.223 to 10.36±0.68 days respectively in case of 0,2,4,6 and 8 days pre-refrigerated cocoon. The trend of increase in the pupal duration due to increased duration of cold storage of cocoon is almost similar in all the cases of 0, 2, 4, 6 and 8 days of pre-refrigeration. The maximum pupal duration 11.33±0.046 days was noticed in case of 2 days pre-refrigerated-30 day refrigerated cocoon while minimum pupal duration 7.89±0.35 days were noticed for non refrigerated cocoon. Two way ANOVA indicates that both, the duration of refrigeration and pre-refrigeration period have no significant influence on the pupal duration of *Bombyx mori*.

Survival of pupae: The data presented in table 3 clearly indicates that both, the duration of refrigeration and pre-refrigeration period of cocoon influenced the survival of pupae. At all the condition of pre-refrigeration period (0, 2, 4, 6 and 8 days), the survival percentage of pupae decreased with the increasing duration of refrigeration. With the increasing duration of cold storage of cocoon from 0 to 30 days, the survival percent of pupae reduced sharply from 82.21 ± 1.93 to the level of 72.88±0.443%, 83.10±1.176 to 61.32±1.33%, 84.44 ±1.78 to 63.10±1.935%, 85.33 ± 1.54 to 55.10±1.776% and 80.44±0.44 to 68.44±0.89% for the pre-refrigeration period of 0, 2, 4, 6 and 8 days respectively. At all the conditions of pre-refrigeration period, good survival percentage of pupae has been noticed. The maximum percentage of survival of pupae (85.33±1.54%) was noticed in case of 6 day pre-refrigerated-0 day refrigerated cocoons and the minimum percent of survival of pupae (55.10± 1.77%) was noticed in 6 days pre-refrigerated-20 days refrigerated cocoon. Two way ANOVA indicates that both, the duration of refrigeration and pre-refrigeration have significant (P<0.01) influence on the survival of pupae of *Bombyx mori.*

DISCUSSION

Pupal weight: The pupal weight of *Bombyx mori* has been noticed to be influenced by the variation in the level of secreted hormones (Khan et al,1997) and genotype variation (Rajashekhar Gouda et al.,1997; Rajanna and Puttaraju,1998).The difference between the weight of cocoon and shell is the weight of the pupa (Gaviria et al.,2006).The mature larvae and pupae of *Bombyx mori* weighed more when reared at 25°C in comparison to be reared at 30°C (Verma and Atwal,1968; Ali et al.,1990) and similarly (Gaur and Upadhyay, 2002) at 80±5%RH and at 12±1 hrs light a day. The refrigeration of silkworm eggs significantly influenced the pupal weight of *Bombyx mori* (Vishwewara Gowda et al.,1987) .The pupal weight varies in accordance with the rearing conditions and the processing during the grainage operation performed by the chilling of eggs (Pandey and Upadhyay 1999).The cold storage of silkworm eggs considerably influenced the pupal weight of silkworm but the pre-refrigeration period of eggs has no appreciable impact on the pupal weight (Pandey and Upadhyay, 2001). The silkworm larvae, exposed to 14 hrs light a day caused an increase in the pupal weight (Janarthan et al., 1994).The exposure of *Bombyx mori* larvae in the magnetic field of 3500 gauss at various times caused an increase in the weight of larvae and pupae (Chaugale and More, 1992). Magnetization of *Bombyx mori* eggs caused improvement in the pupal weight was exposed to 3000 gauss magnetic strength (Tripathi and Upadhyay, 2006).Topical application of methoprene ,juvenile hormone analogues positively influenced the pupal development, resulted in the good weight of pupae (Miranda et al., 2002) The puparial length increased as puparial weight increased (Alfredo et al., 2006).A juvenile hormone mimic R394, when topically applied on the abdominal tergum of silkworm to improved the larval and pupal performance (Gangwar, 2009). Marked variations were found in respect of pupal weight rearing on fountain tree. (Patil et al., 2008).

Pupal duration: The pupal duration of *Bombyx mori* was influenced by the varieties of mulberry, given as food to the larvae (Bheemnna et al., 1989) and certain type of hormone (Khan et al., 1997). Variation in refrigeration period of silkworm eggs caused considerable influence on the pupal duration of *Bombyx mori.* (Pandey and Upadhyay, 2002). Juvenile hormone secreted by corpora allota is responsible for preventing metamorphosis (Baker, 2003). On the treatment with synthetic juvenoid R 394, the prolongation in the pupal duration was noticed to be proportional to the body weight (Nair et al., 2004). A juvenile hormone mimic R394, when topically applied on the abdomen tergum of silkworm, improved the pupal duration (Gangwar, 2009). Pupal period was prolonged up to a maximum of 68 days at 7.5°C (RajKhowa et al., 2011). Different periods of cold storage (in days) effect the pupal duration (Mona M. Mahmoud , 2013). Difference in pupal duration on different host plant might be due to the optimum temperature, relative humidity, maintained during rearing period and it also indicated that hosts did not exhibit any influence on pupal period (Manjunathanaik et al ., 2010). Environmental conditions during embryonic development not only affect the diapauses nature of eggs but also pupal duration (Kai et al., 1971).

Survival of pupae: The survival and development of insects are at the mercy of nature and developmental activities are restricted in accordance with the prevailing ecological conditions and to a certain extent to their genetic built up (Andrewartha and Birch, 1954; Robertson, 1957 and Krishnaswamy, 1973). Opined that certain physiological alterations influenced the rate of pupal survival in *Bombyx mori*.(Upadhyay and Pandey 2001). High temperature during the later developmental stage considerably reduced the pupation and survival rate of pupae (Venugopal and Krishnaswami, 1987). Survival of pupae in succeeding generation was maximum in the progeny obtained from

heaviest female pupae (Govindan et al., 1990). The oral administration of folic acid during 5th instar silkworm significantly influenced the survival percent of silkworm (Rahmathulla et al., 2007; Rai et al., 2002). The survival rate of pupae was effected by refrigeration and pre-refrigeration period (Upadhyay et al., 2009).

Thus it may be concluded from the data that the chilling duration and pre-refrigeration period of cocoon may cause certain physiological alterations which influenced the pupal performance in terms of pupal weight, pupal duration and survival of pupae of *B. mori*.

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Table 1. Effect of the refrigeration of cocoon on the weight (gm) of *Bombyx mori* pupae.

Pre-refrigeration Period(days)	Refrigeration Period (days)							F_1-ratio $n_1=6$
	0	5	10	15	20	25	30	
0	0.8687 ±0.039	0.8105 ±0.02	0.7649 ±0.01	0.7421 ±0.004	0.7122 ±0.007	0.6809 ±0.005	0.65 ±0.01	
2	0.8728 ±0.041	0.7809 ±0.035	0.7412 ±0.008	0.7253 ±0.009	0.7002 ±0.007	0.6643 ±0.005	0.6409 ±0.009	
4	0.8789 ±0.012	0.7305 ±0.005	0.7254 ±0.009	0.7007 ±0.009	0.6647 ±0.007	0.6309 ±0.012	N.Sd	
6	0.8809 ±0.02	0.7055 ±0.008	0.6804 ±0.008	0.6806 ±0.010	0.6226 ±0.008	N.Sd	N.Sd	**5.7690***
8	0.9074 ±0.023	0.6908 ±0.002	0.6619 ±0.008	N.Sd	N.Sd	N.Sd	N.Sd	

F_2-ratio= 5.4335* **$n_2=4$**

*P<0.01 N.Sd=Not survived

Each value represents mean ± S.E. of three replicates

Table 2. Effect of the refrigeration of cocoon on the pupal duration (days) of *Bombyx mori*

Pre-refrigeration Period(days)	Refrigeration Period (days)							F_1-ratio $n_1=6$
	0	5	10	15	20	25	30	
0	7.89 ±0.35	8.2 ±0.08	8.41 ±0.33	8.9 ±0.26	9.26 ±0.051	9.78 ±0.313	10.01 ±0.36	
2	8.19 ±0.09	8.88 ±0.25	8.84 ±0.21	9.55 ±0.269	9.87 ±0.251	10.59 ±0.243	11.33 ±0.046	
4	8.22 ±0.112	9.12 ±0.023	9.64 ±0.256	9.81 ±0.264	10.55 ±0.245	11.31 ±0.048	N.Sd	**1.245***
6	8.47 ±0.266	9.33 ±0.023	9.9 ±0.319	10.3 ±0.035	10.92 ±0.3	N.Sd	N.Sd	
8	8.77 ±0.0223	9.88 ±0.557	10.36 ±0.68	N.Sd	N.Sd	N.Sd	N.Sd	

F_2-ratio= 5.4335* **$n_2=4$**

*P<0.01 N.Sd=Not survived

Each value represents mean ± S.E. of three replicates

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Table 3. Effect of the refrigeration of cocoon on the survival (%) of *Bombyx mori* pupae.

Pre-refrigeration Period(days)	Refrigeration Period (days)							F_1-ratio $n_1=6$
	0	5	10	15	20	25	30	
0	82.21 ±1.93	83.11 ±1.60	82.66 ±0.77	79.1 ±0.44	77.77 ±0.44	73.99 ±1.38	72.88 ±0.44	
2	83.1 ±1.176	80.44 ±1.175	77.77 ±1.176	76.88 ±0.443	71.11 ±0.890	70.22 ±1.78	61.32 ±1.33	
4	84.44 ±1.78	78.66 ±0.77	75.99 ±1.33	74.22 ±0.89	68.44 ±0.89	63.1 ±1.935	N.sd	**6.1739***
6	85.33 ±1.54	75.11 ±1.60	72.44 ±1.60	71.55 ±1.176	55.1 ±1.77	N.sd	N.sd	
8	80.44 ±0.44	72.88 ±1.938	68.44 ±0.89	N.sd	N.sd	N.sd	N.sd	

F_2-ratio= 5.4335* **$n_2=4$**

*P<0.01 N.Sd=Not survived

Each value represents mean ± S.E. of three replicates