4. RESULTS

4.1 Embryonic development of Muga silkworm, *A.assama*

4.1.1 Detailed embryonic development of Muga silkworm, *A.assama* under normal condition

4.1.1.1 Embryonic development at 6 hours

4.1.1.1.1 Embryonic development at 6 hours after oviposition under Microscope

4.1.1.1.1.1 Embryonic development at 6 hours after oviposition under Stereo Binocular Microscope

After 6 hours of egg deposition cleavage nuclei were migrated to the surface of the egg and arranged spherically (Plate1A) starting a stage of de-lamination.

4.1.1.1.1.2 Embryonic development at 6 hours after oviposition under Scanning Electron Microscope

The sphere had swelled at the micropyle end and at least at opposite end of egg (Plate 2A).

4.1.1.1.2 Embryonic development after 6 hours incubation through histological sections

These one cell layered epithelium was differentiated into blastoderm (Plate 5A).

4.1.1.2 Embryonic development at 12 hours

4.1.1.2.1 Embryonic development at 12 hours after oviposition under Microscope

4.1.1.2.1.1 Embryonic development at 12 hours after oviposition under Stereo Binocular Microscope

After 12 hours of egg deposition, blastomeres were rapidly increased to develop blastoderm which was very thin in the dorsal site (Plate1B) and thick at the ventral site.

4.1.1.2.1.2 Embryonic development at 12 hours after oviposition under Scanning Electron Microscope

Ventral plate was emerged through condensation, both from the cephalic and caudal end (Plate 2B). Microvilli like projections were evident on blastoderm.

4.1.1.2.2 Embryonic development after 12 hours incubation through histological sections

Basement membrane had separated blastomeres from vitellophages, accumulated through endocytosis activity. Germ cells were appeared in the posterior pole outside blastoderm. On yolk mass outer yolk cells were distinct (Plate 5B).

Soon after formation of blastoderm, gradual invagination inside egg was initated. Amniotic fold appeared with inner and outer layer which was transformed later into chorion and amnion, respectively. Serosa having flattened cell, formation was completed by this time. Amnion covers germ band (Plate 5C). Constriction to ventral plate gives rise to germ band (Plate2B, Plate 5D). Germ band already under gone gastrulation and differentiate into ectoderm. Also pseudo-startified lanceolate cells were evident.

4.1.1.3 Embryonic development at 24 hours

4.1.1.3.1 Embryonic development at 24 hours after oviposition under Microscope

4.1.1.3.1.1 Embryonic development at 24 hours after oviposition under Stereo Binocular Microscope

After 24 hour, muga silk worm egg germ band had appeared with proctocephalon and protocrom (Plate 1C).

4.1.1.3.1.2 Embryonic development at 24 hours after oviposition under Scanning Electron Microscope

Soon after attaining ‘daruma’ stage, primitive groove appeared along the median line of the germ band surface, which was narrow at the centre and wider at two edges (Plate 2C).

4.1.1.3.2 Embryonic development after 24 hours incubation through histological sections

This stage had resembled with ‘kokeshi’ of *Bombyx* sp. Segmentation was initiated during this stage.

4.1.1.4 Embryonic development at 48 hours

4.1.1.4.1 Embryonic development at 48 hours after oviposition under Microscope

4.1.1.4.1.1 Embryonic development at 48 hours after oviposition under Stereo Binocular Microscope

After 48 hours, germ band had appeared around the egg periphery (Plate 1D).

4.1.1.4.1.2 Embryonic development at 48 hours after oviposition under Scanning Electron Microscope

Distinct germ band had covered the yolk over almost entire length of the egg (Plate 2D).

4.1.1.4.2 Embryonic development after 48 hours incubation through histological sections

After 48 hours, amniotic membrane was extended along the whole length of embryo by elongated flattened cells. The ectoderm had evolved multi-stratified layer with elongated cells (Plate 6, A &B). Fluid filled amniotic cavity became distinct. Serosa had covered just under the chorion surrounding embryo, amnion and yolk and presence of two membranes were called synapomorphy.

4.1.1.5 Embryonic development at 72 hours

4.1.1.5.1 Embryonic development at 72 hours after oviposition under Microscope

4.1.1.5.1.1 Embryonic development at 72hours after oviposition under Stereo Binocular Microscope

As development continued 72 hours embryo had started blastokinesis soon after formation of germ band (Plate 1 E).

4.1.1.5.1.2 Embryonic development at 72hours after oviposition under Scanning Electron Microscope

Blastokinesis had entailed early entry and later exit of embryo from yolk as vivid in Plate 2 E.

4.1.1.5.2 Embryonic development after 72 hours incubation through histological sections

Blastokinesis had two types of movements *i.e.* anatrepsis (upward) and ketatrepsis (down ward) were shown in Plate 6C&D. During anatrepsis, entry of tissues into yolk was evident. Embryo extends in length and segmentation and appendage formation initiated. The ectoderm was crossed by transverse furrows, limited different segments and differentiated polygonal neuroblast with clear edges and neurotic projections. Neural groove also appeared during this stage (Plate6 D). Protuberance had appeared in thoracic segment. At the later stage protuberance appeared in thoracic segments also and continued to appear in abdominal segments.

4.1.1.6 Embryonic development at 96 hours

4.1.1.6.1 Embryonic development at 96 hours after oviposition under Microscope

4.1.1.6.1.1 Embryonic development at 96 hours after oviposition under Stereo Binocular Microscope

In 96 hours embryo (Plate 1 F) katatrepsis was initiated as evasion or outward movement of both embryo and amnion. After blastokinesis egg again had emerged on the surface of egg.

4.1.1.6.1.2 Embryonic development at 96hours after oviposition under Scanning Electron Microscope

During progressive emergence head and antennae, the legs and lastly abdomen was released from yolk (Plate 2F&3B).

4.1.1.6.2 Embryonic development after 96 hours incubation through histological sections

A back flip or 180˚ revolution of embryo was observed (Plate 6 E & F). A pair of labial protuberance became distinct in front of head fold. Stomodeum and proctodeum had appeared and gradually become tubular.

In the thoracic region rudiments of appendages had appeared and in cephalic region, formed by the beginning of stomodaeum. The differentiation of labrum, occurred in protocephalon, over which yolk was present.

In the 96 hours egg, the caudal area was surrounded by the amino proctodeal cavity bounded by amnion and then invaginate to originate proctodaeum, along with coelomic cavities, behind which germ cells are clustered. In the proctocephalon there were two coelomic cavities. Amniotic cavity had enlarged and the amnion surrounding the embryo and yolk. Serosa had secreted a distinct culticular layer. Here segmentation was more advanced. In the protocorm, the buds of the gnathal appendages, of the three pairs of the legs and of the ten abdominal segments were evident. In the first abdominal segment there was pleuropodium of conical shaped and small structure. In the following seven segments and in the tenth there were proleg buds. The ventral nerve cord was well defined.

4.1.1.7 Embryonic development at 120 hours

4.1.1.7.1 Embryonic development at 120 hours after oviposition under Microscope

4.1.1.7.1.1 Embryonic development at 120 hours after oviposition under Stereo Binocular Microscope

During 120 hours embryo (Plate1G), length of embryo had covered almost whole length of the embryo and both end came in contact which was called dorsal closure.

4.1.1.7.1.2 Embryonic development at 120 hours after oviposition under Scanning Electron Microscope

Dorsal closure was distinctly vivid after utilization of yolk along with extra embryonic membranes (Plate 2G).

4.1.1.7.2 Embryonic development after 120 hours incubation through histological sections

Soon after dorsal closure involution was started to move embryo from ventral side to dorsal side (Plate7A). Vertical turning of posterior abdominal segment had placed the abdominal region in a straight line. Then abdominal region reached towards anterior region at the level of prothorax. Fore gut and hind gut had differentiated from anterior and posterior ectodermal invagination respectively (Plate7B, C, D).

4.1.1.8 Embryonic development at 6 hours

4.1.1.8.1 Embryonic development at 6 hours after oviposition under Microscope

4.1.1.8.1.1 Embryonic development at 6 hours after oviposition under Stereo Binocular Microscope

After 144 hours (Plate1H), head capsule formation was completed and mouth parts became mature.

4.1.1.8.1.2 Embryonic development at 6 hours after oviposition under Scanning Electron Microscope

Three segmented antennae with antennal setae, mandibles and labrum were well developed.

4.1.1.8.2 Embryonic development after 6 hours incubation through histological sections

Tips of labrum and labium became segmented (Plate7E, F). Thoracic legs became segmented with claws at distal end. Rudiments of setae had developed on body surface.

4.1.1.9 Embryonic development at 168 hours

4.1.1.9.1 Embryonic development at 168 hours after oviposition under Microscope

4.1.1.9.1.1 Embryonic development at 168 hours after oviposition under Stereo Binocular Microscope

Entire body of 168 hours embryo (Plate 1I) had covered with strong setae and embryonic moult was occurred in this stage.

4.1.1.9.1.2 Embryonic development at 168 hours after oviposition under Scanning Electron Microscope

Caudal horns appeared in this stage. Mandibles became sclerotised and pigmented at the distal end. Larval eyes (i.e. Ocelli) appear as six brown spot on either side of head. The spiracles were clearly visible on the sides of body. Head capsule and mouth appendages were sclerotised and well pigmented. Rectal sac became distinct (Plate 4 A, B,C,D).

4.1.1.9.2 Embryonic development after 168 hours incubation through histological sections

The amnion and serosa disappeared by fragmentation. Embryo had ingested the embryonic membranes and sensitive for adverse environmental condition. Entire body of embryo became sclerotised.

4.1.2. Effect of low temperature stress on embryonic development:

4.1.2.1 Effect of low temperature stress on 12 hours embryo

After 3days refrigeration, 12 hours of eggs, embryo became larger in size. Germ band became thick (Plate11A&B).

4.1.2.2 Effect of low temperature stress on 24 hours embryo

After 3days refrigeration 24 hour muga silk worm eggs became larger, covering the entire length of the egg and were showing resemblance with 48 hour embryos (Plate11C&D).

4.1.2.3 Effect of low temperature stress on 48 hours embryo

After 3days refrigeration 48 hours egg was continued to grow in slower rate and were showing resemblance with 96hours embryo (Plate E&F).

4.1.2.4 Effect of low temperature stress on 72 hr embryo

As development continues slowly 72 hours embryo after 3 days refrigeration had shown resemblance with 120 hr embryo (Plate G&H). However electron microscopic studies and histological studies confirm similar stage specific characteristics.

4.2 Effect of temperature stress on different embryonic stages

To identify low temperature resistant embryonic stages from 24 hours to 144 hours embryo in eleven treatments (*viz.* 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144 hours) under four temperature shocks (4, 6, 8, 10±1˚C) for ten days were taken for hatching percentage and incubation periods. Data thus obtained were recorded and analyzed statistically.

4.2.1 Effect of temperature stress on different embryonic stages on hatching percentage

4.2.1.1. Effect of temperature stress at 4±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (90.85%) followed non-significantly by 36 hours (89.85%), 48 hours (89.69%), 60 hours (89.37%), 72 hours (89.36%) and control (82%) and significantly by 84 hours (67.23%) , 96 hours (59.94%), 108 hours (57.96%) having non significant variation between 84 hours to 108 hours and others. Lowest hatching was observed at 144 hours(44.29%) having non significant variation with 120 hours (66.46%) and 132 hours (45.34%) (Table 1)

Table1. Effect of low temperature stress at 4±1˚C for different embryonic stages on hatching percentage

Embryonic Stage	Hatching percentage
24hours	90.85±0.2
36hours	89.85±0.22
48hours	89.69±0.24
60hours	89.37±0.62
72hours	89.36±0.47
84hours	67.23±0.34
96hours	59.94±0.37
108hours	57.96±0.50
120hours	46.46±0.12
132hours	45.34±0.99
144hours	44.29±0.43
Control	82.00±0.05
CD at 5%	9.52

4.2.1.2. Effect of temperature stress at 6±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (89.79%) followed non significantly by 36hours (89.75%), 48 hours (89.58%), 60 hours (89.53%), 72 hours (88.93%) and control (82%) and significantly by 84 hours (59.96%) and 96 hours (46.93%) having non significant variation between the two. Lowest hatching percentage was recorded at 144 hours (18.67%) increased non significantly at 132 hours (22.36%) and 120 hours (25.12%) (Table2).

Table2. Effect of low temperature stress at 6±1˚C for different embryonic stages on hatching percentage

Embryonic Stage	Hatching percentage
24hours	89.79±0.189
36hours	89.75±0.34
48hours	89.58±0.55
60hours	89.53±0.46
72hours	88.93±0.26
84hours	59.96±0.78
96hours	46.93±0.53
108hours	39.74±0.07
120hours	25.124±0.1
132hours	22.36±0.56
144hours	18.676±0.91
Control	82.00±0.05
CD at 5%	18.73

4.2.1.3. Effect of temperature stress at 8±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (89.58%) followed by non significant changes by 36 hours (89.71%), 48 hours (77.74%) and control (82%) and significantly by 60 hours (61.70%), 72 hours (43.59%), 84 hours (39.32%) having non significant variation between them and followed significantly by 96 hours (32.04%). No hatching was recorded at108hours, 120hours, 132hours and 144hours (Table 3).

Table3. Effect of low temperature stress at 8±1˚C for different embryonic stages on hatching percentage

Embryonic Stage	Hatching percentage
24hours	89.58±0.37
36hours	89.71±0.07
48hours	77.74±0.64
60hours	61.70±0.14
72hours	43.59±0.19
84hours	39.32±0.44
96hours	32.04±0.88
108hours	0.0000
120hours	0.0000
132hours	0.0000
144hours	0.000
Control	82.00±0.05
CD at 5%	24.47

4.2.1.4. Effect of temperature stress at 10±1˚C on different embryonic stages on hatching percentage

Hatching percentage was highest at 24 hours (83.51%) followed non significantly by 36 hours (79.41%), 48 hours (64.76%) and control (82%) and followed significantly by 60 hours (45.95%), 72 hours (39.94%) having non significant variation between the two. (Table 4)

Table 4. Effect of low temperature stress at 10±1˚C for different embryonic stages on hatching percentage

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Embryonic Stage	Hatching percentage
24 hours	83.51±0.76
36 hours	79.41±0.81
48 hours	64.76±0.73
60 hours	45.95±0.66
72 hours	39.94±0.28
84 hours	0.0000
96 hours	0.0000
108 hours	0.0000
120 hours	0.0000
132 hours	0.0000
144 hours	0.000
Control	82.00±0.05
CD at 5%	22.51

4.2.1.5. Combined effect of different temperature stresses and different embryonic stages on hatching percentage

24 hours embryo at 4±1˚C showed highest hatching percentage (90.85%) having non significant variation with the same embryonic stage at 6±1˚C(89.79%) and 8±1˚C(89.58%); 36 hours embryonic stages at 4±1˚C(89.85%), 6±1˚C(89.75%), 8±1˚C(89.71%); 48 hours embryonic stages at 4±1˚C(89.69%), 6±1˚C(89.58%), 60 hours at 4±1˚C (89.37%),6±1˚C (89.53%) and 72 hours embryonic stages at 4±1˚C(89.36%), 6±1˚C (88.93%) and significantly by 24 hours embryo at 10±1˚C (83.51%) and by others (Table 5).

So it was observed that control treatments and the embryonic stages up to 72 hours when treated with 4±1˚ and 6±1˚, temperature stress showed non significant variation in hatching percentage. As the temperature shock decreased to 8±1˚C or 10±1˚ the hatching percentage was also decreased but still close to control up to 48 hours of embryonic stage.

Moreover no hatching from 84 hours onwards embryonic ages was found at 10±1˚C from 108 hour embryonic stage at 8±1˚C (Table 5).

Table 5. Combined effect of different low temperature stresses for different embryonic stages on hatching percentage

	4±1˚C	6±1˚C	8±1˚C	10±1˚C
24h	90.85±0.2	89.79±0.19	89.58±0.37	83.51±0.76
36h	89.85±0.22	89.75±0.34	89.71±0.07	79.41±0.81
48h	89.69±0.24	89.58±0.55	77.74±0.64	64.76±0.73
60h	89.37±0.62	89.53±0.46	61.70±0.14	45.95±0.66
72h	89.36±0.47	88.93±0.26	43.59±0.19	39.94±0.28
84h	67.23±0.34	59.96±0.78	39.32±0.44	0.0000
96h	59.94±0.37	46.93±0.53	32.04±0.88	0.0000
108h	57.96±0.50	39.74±0.07	0.0000	0.0000
120h	46.46±0.12	25.124±0.1	0.0000	0.0000
132h	45.34±0.99	22.36±0.56	0.0000	0.0000
144h	44.29±0.43	18.676±0.91	0.0000	0.0000
CD at 5%	3.07			

4.2.2 Effect of different temperature stress on incubation period of different embryonic stages

4.2.2.1 Effect of temperature stress at 4±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 108 hours (18.72 days) non significantly followed by 120 hours (18.66 days), 132 hours (18.62 days) and144 hours (18.60 days) and followed significantly by 60 hours (18.17 days), 48 hours (18.16 days), 72 hours (18.14 days),84 hours (18.04 days) and 96 hours (18 days). Lowest incubation period was observed from 24 hours (16.96 days) followed significantly by 36 hours (17.98 days). Incubation period was significantly higher in all the treatments over control (7days) (Table 6).

Table 6. Effect of low temperature stress at 4±1˚C for different embryonic stages on incubation period (days)

Embryonic Stage	Incubation Period(days)
24h	16.96±0.31
36h	17.58±0.09
48h	18.16±0.54
60h	18.17±0.56
72h	18.14±0.62
84h	18.04±0.62
96h	18.00±0.67
108h	18.72±0.75
120h	18.66±0.81
132h	18.62±0.87
144h	18.60±0.95
Control	7±0.03
CD at 5%	0.31

4.2.2.2 Effect of temperature stress at 6±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 48 hours (18.14 days) non significantly followed by 72 hours (18.13 days), 84 hours (17.86 days), 60 hours (17.75 days), 96 hours (17.43 days), 36 hours (17.42 days) and 24 hours (17.08 days). Lowest incubation period was observed at 144hours (11.02 days) and non significantly followed by 132hours (11.03 days) and 120hours (12.83 days) embryo. Incubation period was significantly higher in all the treatments over control (7days) (Table 7).

Table 7. Effect of low temperature stress at 6±1˚C for different embryonic stages on incubation period (days)

Embryonic Stage	Incubation Period (days)
24h	17.08±0.35
36h	17.42±0.59
48h	18.14±0.97
60h	17.75±0.20
72h	18.13±0.52
84h	17.86±0.34
96h	17.43±0.25
108h	16.06±0.26
120h	12.83±0.94
132h	11.03±0.35
144h	11.02±0.36
CD at 5%	1.50

4.2.2.3 Effect of temperature stress at 8±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 24 hours (16.95days), non significantly followed by 60 hours (16.92 days), 48 hours (16.77days), 36 hours (16.55days), 72 hours (15.21days), 84 hours (15.12 days) and 96 hours (15.04 days) and significantly by control (7days). As no hatching was observed from 108 hours, zero incubation days recorded. Incubation period was significantly higher in all the treatments over control (7days) till 96 hours(Table 8).

Table 8. Effect of low temperature stress at 8±1˚C for different embryonic stages on Incubation period (days)

Embryonic Stage	Incubation Period (days)
24h	16.95±0.09
36h	16.55±0.5
48h	16.77±0.36
60h	16.92±0.62
72h	15.21±0.88
84h	15.12±0.92
96h	15.04±0.97
108h	0
120h	0
132h	0
144h	0
Control	7±0.03
CD at 5%	4.81

4.2.2.4 Effect of temperature stress at 10±1˚C on incubation period of different embryonic stages

Highest incubation period was recorded at 24 hours (16.93 days) non significantly followed by 36 hours (16.48 days), 48 hours and 60 hours (16.39 days) and 72 hours (15.01 days). As no hatching was observed from 84 hours, zero incubation days were recorded. Incubation period was significantly higher in all the treatments over control (7days) till 72 hours(Table 9)

Table 9. Effect of low temperature stress at 10±1˚C for different embryonic stages on incubation period (days)

Embryonic Stage	Incubation Period (days)
24h	16.93±0.05
36h	16.48±0.87
48h	16.39±0.07
60h	16.39±0.34
72h	15.01±0.04
84h	0
96h	0
108h	0
120h	0
132h	0
144h	0
Control	7±0.03
CD at 5%	5.09

4.2.2.5 Combined effect of different temperature stresses and different embryonic stages on incubation period

It revealed that highest incubation period was observed at 4±1˚C low temperature stress, by108 hours (18.72 days), non significantly followed by 4±1˚C x 120 hours (18.66 days), 4±1˚C x 132 hours (18.62 days) and 4±1˚C x 144 hours (18.60 days) and significantly by 4±1˚C x 60hours (18.17 days), 4±1˚C x 48hours (18.16 days), 4±1˚C x 72 hours (18.14 days), 4±1˚C x 84 hours (18.04 days), 6±1˚C x 48 hours (18.14 days) 6±1˚C x 72 hours (18.13 days) having non significant variation among them. Lowest incubation period was observed from 6±1˚C x 144 hours (11.02 days) followed non significantly by 6±1˚C x 132 hours (11.03 days) (Table 10).

Table 10. Combined effect of different low temperature stress for different embryonic stages on incubation period (days)

	4±1˚C	6±1˚C	8±1˚C	10±1˚C
24h	16.96±0.31	17.08±0.35	16.95±0.09	16.93±0.05
36h	17.58±0.09	17.42±0.59	16.55±0.5	16.48±0.87
48h	18.16±0.54	18.14±0.97	16.77±0.36	16.39±0.07
60h	18.17±0.56	17.75±0.20	16.92±0.62	16.39±0.34
72h	18.14±0.62	18.13±0.52	15.21±0.88	15.01±0.04
84h	18.04±0.62	17.86±0.34	15.12±0.92	0
96h	18.00±0.67	17.43±0.25	15.04±0.97	0
108h	18.72±0.75	16.06±0.26	0	0
120h	18.66±0.81	12.83±0.94	0	0
132h	18.62±0.87	11.03±0.35	0	0
144h	18.60±0.95	11.02±0.36	0	0
CD at 5%	0.23			

Finally it can be said that embryonic stages up to 72 hours showed better hatching percentage (90.85 to 88.93%) over control (82%) though the variations were non significant for both low temperature stresses 4±1˚C and 6±1˚C. Incubation period however, was found highest in late embryonic ages only in 4±1˚C, but for 6±1˚C up to 96 hours the incubation periods were longer. But if the embryonic age were considered to be deleted from the actual ten days shock (1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5, 5, 5.5, 6, 6.5 and 7days respectively for 24, 36, 48, 60, 72, 84, 96, 108, 120, 132 and 144hours), then early ages up to 96 hours showed higher incubation periods.

For higher levels of temperature shock that is 8±1˚C and 10±1˚C up to 36 hours old embryo showed higher hatching percentage than control though the variations were non significant. For these two temperatures incubation period were found longer up to 72 hours. For 8±1˚C up to 96 hours can be considered as after that no hatching was observed (Table 10a). Regression analysis also confirmed present findings (Plate 12).

Table 10a. Combined effect of different temperature stress and embryonic age on hatching percentage and incubation period

	4±1˚C		6±1˚C		8±1˚C		10±1˚C	
	H	I	H	I	H	I	H	I
24h	90.85±0.2	16.96±0.31	89.79±0.19	17.08±0.35	89.58±0.37	16.95±0.09	83.51±0.76	16.93±0.05
36h	89.85±0.22	17.58±0.09	89.75±0.34	17.42±0.59	89.71±0.07	16.55±0.5	79.41±0.81	16.48±0.87
48h	89.69±0.24	18.16±0.54	89.58±0.55	18.14±0.97	77.74±0.64	16.77±0.36	64.76±0.73	16.39±0.07
60h	89.37±0.62	18.17±0.56	89.53±0.46	17.75±0.20	61.70±0.14	17.25±0.62	45.95±0.66	16.39±0.34
72h	89.36±0.47	18.14±0.62	88.93±0.26	18.13±0.52	43.59±0.19	15.21±0.88	39.94±0.28	15.01±0.04
84h	67.23±0.34	18.04±0.62	59.96±0.78	17.86±0.34	39.32±0.44	15.12±0.92	0	0
96h	59.94±0.37	18.00±0.67	46.93±0.53	17.43±0.25	32.04±0.88	15.04±0.97	0	0
108h	57.96±0.50	18.72±0.75	39.74±0.07	16.06±0.26	0	0	0	0
120h	46.46±0.12	18.66±0.81	25.124±0.1	12.83±0.94	0	0	0	0
132h	45.34±0.99	18.62±0.87	22.36±0.56	11.03±0.35	0	0	0	0
144h	44.29±0.43	18.60±0.95	18.676±0.91	11.02±0.36	0	0	0	0

So it can be concluded that embryonic stages of 24, 36, 48, 60, 72, 84 and 96 hours can be identified as low temperature resistant embryonic stages, more precisely,up to 96 hours embryonic stages can be explored for improvement of cold preservation technology of seed.

4.3 Effect of different low temperature stress in identified low temperature resistant embryonic stages

The identified low temperature resistant stages namely 24, 36,48, 60, 72, 84 and 96 hours old embryonic stages were given low temperature stresses from 4±1˚C to 10±1˚C with 1˚C interval that is 4±1˚C, 5±1˚C, 6±1˚C, 7±1˚C, 8±1˚C, 9±1˚C and 10±1˚C to determine the optimum low temperature for cold stress for successful low temperature preservation basedon hatching percent and incubation days. Data thus obtained were recorded and analyzed statistically.

4.3.1. Effect of different low temperature stress in identified low temperature resistant embryonic stages on hatching percentage

4.3.1.1. Effect of different low temperature stress in 24 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (90.85%), followed non significantly by 5±1˚C (90.32%), 6±1˚C (89.79%), 7±1˚C (89.69%), 8±1˚C (89.58%) and 9±1˚C (89.54%) and significantly by 10±1˚C (83.51%). Experimental treatments till 10±1˚C (83.51%) had shown better hatching percentage than hatching percent in normal condition (82%) (Table 11).

Table 11. Effect of different low temperature stress on hatching percentage for 24 hours embryonic stage

Temperature	Hatching percentage
4±1˚C	90.85±0.02
5±1˚C	90.32±0.06
6±1˚C	89.79±0.19
7±1˚C	89.69±0.33
8±1˚C	89.58±0.37
9±1˚C	89.54±0.03
10±1˚C	83.51±0.76
CD at 5%	2.69

4.3.1.2. Effect of different low temperature stress in 36 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (89.85%), followed non significantly by 5±1˚C (89.79%), 6±1˚C (89.75%), 7±1˚C (88.23%) and 8±1˚C (87.71%) and significantly by 9±1˚C (82.56%). Lowest hatching percentage was recorded at 10±1˚C (79.41%) having non significant variation with9±1˚C. Temperature stress up to 8±1˚C showed better performance than normal condition (82%). (Table 12)

Table 12. Effect of different low temperature stress on hatching percentage for 36 hours embryonic stage

Temperature	Hatching percentage
4±1˚C	89.85±0.22
5±1˚C	89.79±0.17
6±1˚C	89.75±0.34
7±1˚C	88.23±0.05
8±1˚C	87.71±0.07
9±1˚C	82.56±0.67
10±1˚C	79.41±0.81
CD at 5%	3.18

4.3.1.3 Effect of different low temperature stress in 48 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (89.69%), followed non significantly by 5±1˚C (89.64%) and 6±1˚C (89.58%) and significantly by 7±1˚C (78.72%) and others. Lowest hatching percentage was recorded at 10±1˚C (64.76%) followed significantly by 9±1˚C (72.25%). Temperature stress up to 6±1˚C showed better performance than normal condition (82%).(Table 13)

Table 13. Effect of different low temperature stress on hatching percentage for 48 hours embryonic stage

Temperature	Hatching percentage
4±1˚C	89.69±0.24
5±1˚C	89.64±0.44
6±1˚C	89.58±0.55
7±1˚C	78.72±0.12
8±1˚C	77.74±0.64
9±1˚C	72.25±0.07
10±1˚C	64.76±0.73
CD at 5%	6.91

4.3.1.4 Effect of different low temperature stress in 60 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 5±1˚C (89.59%), followed non significantly by 6±1˚C (89.53%) and 4±1˚C (88.37%) and significantly by 7±1˚C (71.62%) and others. Lowest hatching percentage was recorded at 10±1˚C (45.95%) followed non significantly by 9±1˚C (54.83%) and significantly by others. Temperature stress up to 6±1˚C showed better performance than normal condition (82%). (Table 14)

Table 14. Effect of different low temperature stress on hatching percentage for 60 hours embryonic stage

Temperature	Hatching percentage
4±1˚C	88.37±0.62
5±1˚C	89.59±0.38
6±1˚C	89.53±0.46
7±1˚C	71.62±0.62
8±1˚C	61.70±0.14
9±1˚C	54.83±0.31
10±1˚C	45.95±0.66
CD at 5%	12.93

4.3.1.5 Effect of different low temperature stress in 72 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (89.36%), followed non significantly by 5±1˚C (89.14%) and 6±1˚C (88.93%) and significantly by 7±1˚C (60.48%) and others. Lowest hatching percentage was recorded at 10±1˚C (39.94%) followed non significantly by 9±1˚C (41.77%) and 8±1˚C (43.59%). Temperature stress up to 6±1˚C showed better performance than normal condition (82%). (Table 15)

Table15. Effect of different low temperature stress on hatching percentage for 72 hours embryonic stage

Temperature	Hatching percentage
4±1˚C	89.36±0.47
5±1˚C	89.14±0.09
6±1˚C	88.93±0.26
7±1˚C	60.48±0.51
8±1˚C	43.59±0.19
9±1˚C	41.77±0.16
10±1˚C	39.94±0.28
CD at 5%	16.39

4.3.1.6 Effect of different low temperature stress in 84 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (67.23%), followed non significantly by 5±1˚C (66.6%), 6±1˚C (59.96%) and 7±1˚C (50.64%) and significantly decreased at 8±1˚C (39.32) and 9±1˚C (29.66%). No hatching was recorded at 10±1˚C. All temperature stresses showed poor performances than normal condition (82%). (Table 16)

Table 16. Effect of different low temperature stress on hatching percentage for 84 hours embryonic stage

Temperature	Hatching percentage
4±1˚C	67.23±0.34
5±1˚C	66.6±0.51
6±1˚C	59.96±0.78
7±1˚C	50.64±0.07
8±1˚C	39.32±0.44
9±1˚C	29.66±0.11
10±1˚C	0
CD at 5%	18.75

4.3.1.7 Effect of different low temperature stress in 96 hours embryonic stages on hatching percentage

Highest hatching percentage was observed at 4±1˚C (59.94%), followed non significantly by 5±1˚C (59.43%) and 6±1˚C (46.93%) and significantly by 7±1˚C (39.49%) and 8±1˚C (32.04%). No hatching was recorded at 9±1˚C and 10±1˚C. All temperature stresses showed poor performances than normal condition (82%). (Table 17)

Table 17.Effect of different low temperature stress on hatching percentage for 96 hours embryonic stage

Temperature	Hatching percentage
4±1˚C	59.94±0.37
5±1˚C	59.43±0.06
6±1˚C	46.93±0.53
7±1˚C	39.49±0.56
8±1˚C	32.04±0.88
9±1˚C	0
10±1˚C	0
CD at 5%	19.6

4.3.1.8Combined effect of different low temperature stress on identified low temperature resistant embryonic stages on hatching percentage

Highest hatching % was observedup to9±1˚C for 24 hours, up to 7±1˚C for 36hours and up to 6±1˚C for 48 to 72 hours of embryonic stages having non significant variation among them (90.85 to 88.23%). 36 hours embryonic stage having 8±1˚C temperature stress showed hatching percentage of 87.71% while at 9±1˚C this stage show 82.56% hatching. All these hatching percentages are higher than or at per hatching in normal condition (82%) (Table 18).

Table 18. Combined effect of different low temperature stresses on hatching percentage for identified low temperature resistant embryonic stages

	4±1˚C	5±1˚C	6±1˚C	7±1˚C	8±1˚C	9±1˚C	10±1˚C
24h	90.85±0.2	90.32±0.06	89.79±0.189	89.69±0.33	89.58±0.37	89.54±0.03	83.51±0.76
36h	89.85±0.22	89.79±0.17	89.75±0.34	88.23±0.05	87.71±0.07	82.56±0.67	79.41±0.81
48h	89.69±0.24	89.64±0.44	89.58±0.55	78.72±0.12	77.74±0.64	72.25±0.07	64.76±0.73
60h	89.37±0.62	89.59±0.38	89.53±0.46	71.62±0.62	61.70±0.14	54.83±0.31	45.95±0.66
72h	89.36±0.47	89.14±0.09	88.93±0.26	60.48±0.51	43.59±0.19	41.77±0.16	39.94±0.28
84h	67.23±0.34	66.6±0.51	59.96±0.78	50.64±0.07	39.32±0.44	29.66±0.11	0
96h	59.94±0.37	59.43±0.06	46.93±0.53	39.49±0.56	32.04±0.88	0	0
CD at 5%	2.36						

4.3.2. Effect of different low temperature stress in identified low temperature resistant embryonic stages on incubation period

4.3.2.1 Effect of different low temperature stress in 24 hours embryonic stage on incubation period

Highest incubation period was observed at 6±1˚C (17.08 days) followed significantly by 5±1˚C (17.02 days), 7±1˚C (16.97 days), and 4±1˚C (16.96 days) and others. Lowest incubation period was observed from 10±1˚C (16.93 days). (Table 19)

Table 19. Effect of different low temperature stress on incubation period (Days) for 24 hours embryonic stage

Temperature	Incubation Period (Days)
4±1˚C	16.96±0.31
5±1˚C	17.02±0.17
6±1˚C	17.08±0.35
7±1˚C	16.97±0.03
8±1˚C	16.95±0.09
9±1˚C	16.94±0.51
10±1˚C	16.93±0.05
CD at 5%	0.04

4.3.2.2 Effect of different low temperature stress in 36 hour embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (17.58 days), followed non significantly by 6±1˚C (17.42 days) and 5±1˚C (17.03 days) and followed significantly by 7±1˚C (16.92 days) and others. Lowest incubation period was observed at 10±1˚C (16.48 days) having non significant variation with 9±1˚C (16.59 days), 8±1˚C (16.55 days) and7±1˚C (16.92 days). (Table 20)

Table 20. Effect of different low temperature stress on incubation period (Days) for 36 hours embryonic stage

Temperature	Incubation Period (Days)
4±1˚C	17.58±0.09
5±1˚C	17.03±0.23
6±1˚C	17.42±0.59
7±1˚C	16.92±0.44
8±1˚C	16.55±0.5
9±1˚C	16.59±0.81
10±1˚C	16.48±0.87
CD at 5%	0.56

4.3.2.3 Effect of different low temperature stress in 48 hours embryonic stages on incubation period

Highest incubation period was observed at 5±1˚C (18.17 days), followed non significantly by 4±1˚C (18.16 days), 6±1˚C (18.14 days) and significantly by 7±1˚C (16.90 days) and others. Lowest incubation period was observed at 10±1˚C (16.39 days) having non significant variation with 9±1˚C (16.54 days), 8±1˚C (16.77 days) and 7±1˚C (16.90 days). (Table 21)

Table 21. Effect of different low temperature stress on incubation period (Days) for 48 hours embryonic stage

Temperature	Incubation Period (Days)
4±1˚C	18.16±0.54
5±1˚C	18.17±0.15
6±1˚C	18.14±0.97
7±1˚C	16.90±0.15
8±1˚C	16.77±0.36
9±1˚C	16.54±0.11
10±1˚C	16.39±0.07
CD at 5%	0.71

4.3.2.4 Effect of different low temperature stress in 60 hours embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (18.17 days), followed non significantly by 6±1˚C (17.75 days), 5±1˚C (17.57 days) and followed significantly by 8±1˚C (16.92 days) and others. Lowest incubation period was observed at 10±1˚C (16.39 days) having non significant variation with 9±1˚C (16.82 days), 8±1˚C (16.92 days) and 7±1˚C (16.89 days).(Table 22)

Table 22. Effect of different low temperature stress on incubation period (Days) for 60 hours embryonic stage

Temperature	Incubation Period (Days)
4±1˚C	18.17±0.56
5±1˚C	17.57±0.67
6±1˚C	17.75±0.20
7±1˚C	16.89±0.74
8±1˚C	16.92±0.62
9±1˚C	16.82±0.45
10±1˚C	16.39±0.34
CD at 5%	0.64

4.3.2.5 Effect of different low temperature stress in 72 hours embryonic stages on incubation period

Highest incubation period was observed at 5±1˚C (18.18 days), followed non significantly by 4±1˚C (18.14 days), 6±1˚C (18.13 days) and significantly by 7±1˚C(16.53 days) and others. Lowest incubation period was observed at 10±1˚C (15.01 days) having non significant variation with 9±1˚C (15.16 days), 8±1˚C (15.21 days). (Table 23)

Table 23. Effect of different low temperature stress on incubation period (Days) for 72 hours embryonic stage

Temperature	Incubation Period (Days)
4±1˚C	18.14±0.62
5±1˚C	18.18±0.91
6±1˚C	18.13±0.52
7±1˚C	16.53±0.47
8±1˚C	15.21±0.88
9±1˚C	15.16±0.75
10±1˚C	15.01±0.04
CD at 5%	1.27

4.3.2.6 Effect of different low temperature stress in 84 hours embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (18.04 days) followed non significantly by 6±1˚C (17.86 days) and 5±1˚C (17.65 days) and significantly by7±1˚C (16.09 days) and others. Lowest incubation period was observed at 9±1˚C (13.46 days) At 10±1˚C no hatching was observed. (Table 24)

Table 24. Effect of different low temperature stress on incubation period (Days) for 84 hours embryonic stage

Temperature	Incubation Period (Days)
4±1˚C	18.04±0.62
5±1˚C	17.65±0.01
6±1˚C	17.86±0.34
7±1˚C	16.09±0.13
8±1˚C	15.12±0.92
9±1˚C	13.46±0.21
10±1˚C	0
CD at 5%	1.46

4.3.2.7 Effect of different low temperature stress in 96 hours embryonic stages on incubation period

Highest incubation period was observed at 4±1˚C (18.00 days) followed non significantly by 5±1˚C (17.46 days) and 6±1˚C (17.43 days) and significantly by 7±1˚C (15.66 days) and 8±1˚C (15.04 days). No hatching was observed at 9±1˚C and 10±1˚C (Table 25)

Table 25. Effect of different low temperature stress on incubation period (Days) for 96 hours embryonic stage

Temperature	Incubation Period(Days)
4±1˚C	18.00±0.67
5±1˚C	17.46±0.09
6±1˚C	17.43±0.25
7±1˚C	15.66±0.15
8±1˚C	15.04±0.97
9±1˚C	0
10±1˚C	0
CD at 5%	1.13

4.3.2.8 Combined effect of different low temperature stress on identified low temperature resistant embryonic stages on incubation period

Highest incubation period was observed from 4±1˚C temperature stress on 48 hours to 96 hours embryo (18.0 to 18.17 days); 48 and 72 hours at both 5±1˚C and 6±1˚C temperature stress (18.13 to 18.18 days) followed significantly by 60 hours (16.96 days) and 84 hours(17.86 days) embryo at 6±1˚C and also significantly with 60 hours (17.57 days) and 84 hours (17.65 days) egg at 5±1˚C and 36 hours embryo at 4±1˚C(17.58 days) having non significant variation among them. More over from 7±1˚C to higher range, performance were poor and even at 10±1˚C, hatching was not observed beyond 72 hours.(Table 34)

Table 26. Combined effect of different low temperature stresses on incubation period for identified low temperature resistant embryonic stages

	4±1˚C	5±1˚C	6±1˚C	7±1˚C	8±1˚C	9±1˚C	10±1˚C
24h	16.96±0.31	17.02±0.17	17.08±0.35	16.99±0.03	16.95±0.09	16.94±0.51	16.93±0.05
36h	17.58±0.09	17.03±0.23	17.42±0.59	16.97±0.44	16.55±0.5	16.59±0.81	16.48±0.87
48h	18.16±0.54	18.17±0.15	18.14±0.97	16.94±0.15	16.77±0.36	16.54±0.11	16.39±0.07
60h	18.17±0.56	17.57±0.67	16.96±0.20	16.93±0.74	16.92±0.62	16.42±0.45	16.39±0.34
72h	18.14±0.62	18.18±0.07	18.13±0.52	16.53±0.47	15.21±0.88	15.16±0.75	15.01±0.04
84h	18.04±0.62	17.65±0.01	17.86±0.34	16.09±0.13	15.12±0.92	13.46±0.21	0
96h	18.00±0.67	17.46±0.16	17.43±0.25	15.66±0.15	15.04±0.97	0	0
CD at 5%	0.19						

Again it was found that embryonic stages up to 72 hours performed better when the temperature stress was high **(**4±1˚C to 6±1˚C) when compared to normal condition. Though, early embryonic stages could survive well even in 10±1˚C also. More over it was observed that 60% hatching could be done up to 96 hour when treated with very low cold shock.

Similarly incubation days become shorter in older embryos with the increase of temperature. However, very early embryos showed longer incubation days even in 10±1˚C (Table 27). Regression analysis also confirmed present findings (Plate 13).

Table 27. Combined effect of different temperature stress and identified low temperature resistant embryonic age on hatching percentage and incubation period

	4±1˚C		5±1˚C		6±1˚C		7±1˚C		8±1˚C		9±1˚C		10±1˚C	
	H	I	H	I	H	I	H	I	H	I	H	I	H	I
24h	90.85 ±0.2	17.18 ±0.31	90.32 ±0.06	17.26 ±0.17	89.79± 0.189	17.33 ±0.35	95.69 ±0.33	16.79 ±0.03	99.58 ±0.37	16.25 ±0.09	93.54 ±0.03	17.59 ±0.51	93.51 ±0.76	18.93 ±0.05
36h	89.85 ±0.22	17.58 ±0.09	89.3± 0.17	17.03 ±0.23	84.75± 0.34	16.48 ±0.59	88.23 ±0.05	17.52 ±0.44	89.71 ±0.07	18.55 ±0.5	82.56 ±0.67	17.99 ±0.81	79.41 ±0.81	17.42 ±0.87
48h	89.58 ±0.24	18.22 ±0.54	89.64 ±0.44	17.31 ±0.55	79.69± 0.55	16.39 ±0.97	78.72 ±0.12	17.3± 0.15	77.74 ±0.64	18.20 ±0.36	72.25 ±0.07	17.49 ±0.11	64.76 ±0.73	16.77 ±0.07
60h	88.37 ±0.62	18.17 ±0.56	88.95 ±0.38	17.57 ±0.67	79.53± 0.46	16.96 ±0.20	71.62 ±0.62	17.1± 0.74	61.70 ±0.14	17.25 ±0.62	54.83 ±0.31	16.82 ±0.45	45.95 ±0.66	16.39 ±0.34
72h	86.93 ±0.26	18.13 ±0.62	86.14 ±0.09	17.84 ±0.91	77.36± 0.47	17.54 ±0.52	60.48 ±0.51	16.53 ±0.47	43.59 ±0.19	15.51 ±0.88	41.77 ±0.16	15.26 ±0.75	39.94 ±0.28	15.01 ±0.04
84h	67.23 ±0.34	18.44 ±0.62	66.6± 0.51	17.65 ±0.01	59.96± 0.78	16.86 ±0.34	50.64 ±0.07	16.09 ±0.13	39.32 ±0.44	15.32 ±0.92	29.66 ±0.11	13.46 ±0.21	0	0
96h	59.94 ±0.37	18.76 ±0.67	59.43 ±0.06	17.47 ±0.09	46.93± 0.53	16.18 ±0.25	39.49 ±0.56	15.66 ±0.15	32.04 ±0.88	15.14 ±0.97	0	0	0	0

So from overall results, up to 96 hours of embryo could be identified as low temperature resistant embryonic stages excluding the in between hours (36, 60 and 84 hours) of respective day old embryo having non significant variation for better handling and easy identification during large quantity preservation. . And at the same time, low temperatures with in 4±1˚C and 6±1˚C could be applied for determination of optimum low temperature stress period.

4.4. Effect of preservation periods after low temperature stress to the identified embryonic stages

24 , 48, 72 and 96 hours embryonic stages were given 4˚, 5˚ & 6±1˚C temperature stress at different preservation periods namely 3 days, 7 days, 10 days, 15 days and 21 days to determine the optimum periods of low temperature stress. Days delay means the period from egg laying to hatching excluding the preservation days. Data thus obtained were analyzed statistically.

4.4.1 Effect of preservation period after low temperature stress to the identified embryonic stages on hatching percentage

4.4.1.1 Effect of preservation period for 3 days

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (90.92%) having non-significant variation with all the treatments except the hatching percentage of 96 hours old egg at 5˚ & 6±1˚C. (Table 28)

Table 28. Effect of 3 days preservation on hatching percentage of identified embryonic stages at low temperatures

3d	4±1˚C	5±1˚C	6±1˚C
24h	90.92±0.05	90.88±0.25	90.83±0.14
48h	90.65±0.41	90.66±0.04	90.7±0.64
72h	90.63±0.09	90.6±0.23	90.58±0.85
96h	90.54±0.35	90.48±0.15	90.4±0.41
CD at.5%	0.407		

4.4.1.2 Effect of preservation period for 7 days

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (90.9%) having non-significant variation with all the treatments up to 72 hours embryo irrespective of temperature. At 96 hours 7 days preservation showed lower hatching percentage, significantly being lowest from the embryo at 6±1˚C temperature stress.. (Table 29)

Table 29. Effect of 7 days preservation on hatching percentage of identified embryonic stages at low temperatures

7 d	4±1˚C	5±1˚C	6±1˚C
24h	90.9±0.14	90.79±0.17	90.68±0.04
48h	90.56±0.96	90.56±0.55	90.56±0.18
72h	90.54±0.98	90.53±0.55	90.50±0.11
96h	80.16±0.23	77.51±0.37	73.36± 0.45
CD at 5%	3.37		

4.4.1.3 Effect of preservation period for 10 days

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (90.85%) having non-significant variation with all the treatments up to 72 hours embryo irrespective of temperature. At 96 hours 10 days preservation showed significantly poor hatching percentage, lowest being from the embryo at 6±1˚C (43.93%) temperature stress (Table 30)

Table 30. Effect of 10 days preservation on hatching percentage of identified embryonic stages at low temperatures

10 d	4±1˚C	5±1˚C	6±1˚C
24h	90.85±0.2	90.32±0.06	89.79±0.189
48h	89.69±0.24	89.64±0.44	89.58± 0.55
72h	89.36±0.47	89.14±0.09	88.93± 0.26
96h	59.94±0.37	59.43±0.06	46.93± 0.53
CD at 5%	3.81		

4.4.1.4 Effect of preservation period for 15 days on hatching percentage

Highest hatching percentage was recorded on 48 hours embryonic stage at 4±1˚C (89.66%) having non-significant variation with all the treatments up to 72 hours embryo irrespective of temperature. At 96 hours 15 days preservation showed significantly very poor hatching percentage, lowest being from the embryo at 6±1˚C (19.46%) temperature stress (Table 31)

Table 31. Effect of 15 days preservation on hatching percentage of identified embryonic stages at low temperatures

15 d	4±1˚C	5±1˚C	6±1˚C
24h	89.54±0.61	89.58±0.35	89.63±0.8
48h	89.66±0.55	89.45±0.22	89.39±0.29
72h	88.76±0.76	88.69±0.03	88.63±0.59
96h	19.93±0.6	19.51±0.02	19.46±0.44
CD at 5%	5.51		

4.4.1.5 Effect of preservation period for 21 days on hatching percentage

Highest hatching percentage was recorded on 24 hours embryonic stage at 4±1˚C (55.05%) having non-significant variation with 5±1˚C (53.15%) and 6±1˚C (50.1%) and 48 hour embryo at 4±1˚C (49.8%) and significantly by others up to 72 hours old embryo. As no larva hatched, hatching percentage was zero from 96 hour embryo irrespective of temperature stress. (Table 32)

Table 32. Effect of 21days preservation on hatching percentage of identified embryonic stages at low temperatures

21d	4±1˚C	5±1˚C	6±1˚C
24h	55.05±0.11	53.15±0.43	50.1±0.49
48h	49.8±0.59	28.18±0.15	15.16±0.21
72h	39.93±0.52	22.07±0.7	12.1±0.07
96h	0	0	0
CD at 5%	11.13		

4.4.1.6 Combined effect of preservation period, temperature stress and embryonic age on hatching percentage

Highest hatching percentage was observed from 24 hour and 48 hour old embryo when preserved for 3, 7, 10 and 15 days; from 72 hours old embryo for 3,7 and 10 days and also from 96 hours old embryo for 3 days irrespective of temperature stress among 4±1˚C to 6±1˚C (88.93 to 90.9%) significantly followed by 72 hours when preserved for 15 days irrespective of temperatures (88.62 to 88.7%). 96 hours old embryo when preserved for 7 days showed better performance also.(73.30 to 80.10%). Any embryonic age from 24 to 96 hours when preserved for 21 days irrespective of any temperature showed very poor performance even no hatching from 96 hours embryo. (Table 33)

Table 33. Combined effect of preservation period, temperature stress and embryonic age on hatching percentage

Preservation Day	3d			7d			10d			15d			21d		
	4±1 ˚C	5±1 ˚C	6±1 ˚C	4±1 ˚C	5±1 ˚C	6±1 ˚C	4±1˚ C	5±1˚ C	6±1˚ C	4±1 ˚C	5±1 ˚C	6±1 ˚C	4±1 ˚C	5±1 ˚C	6±1 ˚C
24h	90.92±0.05	90.88±0.25	90.83±0.14	90.9±0.14	90.79±0.17	90.68±0.04	90.85±0.2	90.32±0.06	89.79±0.189	89.54±0.61	89.58±0.35	89.63±0.8	55.05±0.11	53.15±0.43	50.1±0.49
48h	90.65±0.41	90.66±0.04	90.7±0.64	90.56±0.96	90.56±0.55	90.56±0.18	89.69±0.24	89.64±0.44	89.58± 0.55	89.66±0.55	89.45±0.22	89 .39± 0.29	49.8±0.59	28.18±0.15	15.16±0.21
72h	90.63±0.09	90.6±0.23	90.58±0.85	90.54±0.98	90.53±0.55	90.50±0.11	89.36±0.47	89.14±0.09	88.93± 0.26	88.76±0.76	88.69±0.03	88.63±0.59	39.93±0.52	22.07±0.7	12.1±0.07
96h	90.54±0.35	90.48±0.15	90.4±0.41	80.16±0.23	77.51±0.37	73.36± 0.45	59.94±0.37	59.43±0.06	46.93± 0.53	19.93±0.6	19.51±0.02	19.46±0.44	0	0	0
CD at 5%	1.972														

4.4.2. Effect of preservation period after low temperature stresses to the identified embryonic stages on hatching days delay

4.4.2.1 Effect of preservation period for 3 days

Longest days delay of hatching was observedby 48 hours embryo irrespective of temperature stress (7.16 to 7.23 days) followed significantly by 24 hours embryo irrespective of temperature stress and by 72 hour embryo at 6±1˚C (7.06 to 7.13 days) followed significantly by others. Shortest days delay was observed from 96 hours embryo (6.32 to 6.40 days) (Table 34)

Table 34. Effect of 3 days preservation on hatching days delay of identified embryonic stages at low temperatures

Preservation Day	3d		
	4±1˚C	5±1˚C	6±1˚C
24h	7.06±0.02	7.08±0.05	7.13±0.03
48h	7.16±0.07	7.22±0.33	7.23±0.26
72h	7.01±0.05	7.09±0.09	7.03±0.25
96h	6.32±0.11	6.37±0.01	6.4±0.15
CD at 5%	0.07		

4.4.2.2 Effect of preservation period for 7 days

Longest days delay of hatching was observed by 48 hours embryo at 4±1˚C (8.13 days) followed non significantly by 24 hours at any temperature (8 to 8.1 days), 72 hour at any temperatures (7.99 to 8.06 days) and 48 hours at 6±1˚C (7.93 days). Significantly shorter days delay was recorded from 96 hours embryo.(Table 35)

Table 35. Effect of 7 days preservation on hatching days delay of identified embryonic stages at low temperatures

Preservation Day	7d		
	4±1˚C	5±1˚C	6±1˚C
24h	8±0.01	8.05±0.04	8.1±0.01
48h	8.13±0.05	7.85±0.19	7.93±0.09
72h	7.99±0.02	8.01±0.41	8.06±0.16
96h	6.5±0.33	6.45±0.05	6.40±0.01
CD at 5%	0.25		

4.4.2.3 Effect of preservation period for 10 days

Longest days delay of hatching was observed by 24 hours and 48 hours embryo irrespective of temperature stress (8.96 to 9.17 days). Significantly followed by 72 hours (8.13 to 8.18 days) and 96 hours (6.43 to 7.0 days) having non significant variation between temperature stress. (Table 36)

Table 36. Effect of 10 days preservation on hatching days delay of identified embryonic stages at low temperatures

Preservation Day	10d		
	4±1˚C	5±1˚C	6±1˚C
24h	8.96±0.01	9.02±0.31	9.08±0.21
48h	9.16±0.04	9.17±0.15	9.14±0.01
72h	8.14±0.01	8.18±0.07	8.13±0.1
96h	7.00±0.41	6.46±0.16	6.43±0.17
CD at 5%	0.28		

4.4.2.4 Effect of preservation period for 15 days

Longest days delay of hatching was observed by 24 hours and 48 hours embryo irrespective of temperature stress, 72 hours and 96 hours at 4±1˚C (3.1 to 3.96 days) and significantly by others. (Table 37)

Table 37. Effect of 15 days preservation on hatching days delay of identified embryonic stages at low temperatures

Preservation Day	15d		
	4±1˚C	5±1˚C	6±1˚C
24h	3.96±0.09	3.95±0.15	3.96±0.05
48h	3.1±0.06	3.28±0.07	3.96±0.01
72h	3.13±0.05	3.08±0.04	3.03±0.05
96h	3.16±0.07	3.09±0.08	3.03±0.07
CD at 5%	0.86		

4.4.2.5 Effect of preservation period for 21 days

96 hours embryo showed no hatching. Among the 24 hours and 72 hours, 24 hours old embryo showed 2.08 days delay at 5±1˚C followed significantly by 6±1˚C (2.03 days) and 4±1˚C (1.96 days). Other treatments showed delay less than one day. (Table 38)

Table 38. Effect of 21 days preservation on hatching days delay of identified embryonic stages at low temperatures

Preservation Day	21d		
	4±1˚C	5±1˚C	6±1˚C
24h	1.96±0.01	2.08±0.01	2.03±0.06
48h	0.64±0.05	0.69±0.04	0.63±0.01
72h	0.34±0.06	0.37±0.15	0.35±0.02
96h	0	0	0
CD at 5%	0.2		

4.4.2.6 Combined effect of preservation period, temperature stress and embryonic age on days delay of hatching

Longest days delay was observed 9.17 days (48 hours embryo at 5±1˚C preserved for 10 days) followed by non significantly by 9.16 days (48 hours embryo at 4±1˚C for 10 days), 9.14 days (48 hours embryo at 6±1˚C for 10 days), 9.08 days (24 hours embryo at 6±1˚C for 10 days), 9.02 days (24 hours embryo at 5±1˚C for 10 days) and significantly followed by 8.96 days (24 hours embryo at 4±1˚C for 10 days), and then non significantly by 8.18 days (72 hours embryo at 5±1˚C for 10 days), 8.14 days(72 hours embryo at 4±1˚C for 10 days), 8.13 days (72 hours embryo at 6±1˚C for 10 days), 8.13 days (48 hours embryo at 4±1˚C for 7 days) , 8.1 days (24 hours embryo at 6±1˚C for 6 days) ,8.06 days(72 hours embryo at 6±1˚C for 7 days),8.05 days (24 hours embryo at 5±1˚C for 7 days) and significantly by 8.01 days (72 hours embryo at 5±1˚C for 7 days), 8 days (24 hours embryo at 4±1˚C for 7 days),7.99 days (72 hours embryo at 4±1˚C for 7 days). (Table 39)

Table 39. Combined effect of preservation period, temperature stress and embryonic age on days delay of hatching

P. Day	3d			7d			10d			15d			21d		
	4±1˚C	5±1˚C	6±1˚C	4±1˚C	5±1˚C	6±1˚C	4±1˚C	5±1˚C	6±1˚C	4±1˚C	5±1˚C	6±1˚C	4±1˚C	5±1˚C	6±1˚C
24h	7.06±0.02	7.08±0.05	7.13±0.03	8±0.01	8.05±0.04	8.1±0.01	8.96±0.01	9.02±0.31	9.08±0.21	3.96±0.09	3.95±0.15	3.96±0.05	1.96±0.01	2.08±0.01	2.03±0.06
48h	7.16±0.07	7.22±0.33	7.23±0.26	8.13±0.05	7.85±0.19	7.93±0.09	9.16±0.04	9.17±0.15	9.14±0.01	3.1±0.06	3.28±0.07	3.96±0.01	0.64±0.05	0.69±0.04	0.63±0.01
72h	7.1±0.05	7.09±0.09	7.03±0.25	7.99±0.02	8.01±0.41	8.06±0.16	8.14±0.01	8.18±0.0 7	8.13±0.1	3.13±0.05	3.08±0.04	3.03±0.05	0.34±0.06	0.37±0.15	0.35±0.02
96h	6.32±0.11	6.37±0.01	6.4±0.15	6.5±0.33	6.45±0.05	6.40±0.01	7.00±0.41	6.46±0.16	6.43±0.17	3.16±0.07	3.09±0.08	3.03±0.07	0	0	0
CD at 5%							0.155								

Table 40 Combined effect of different preservation period on different temperature stress and identified low temperature resistant embryonic age on hatching percentage and days delay of hatching

	3						7						10						15						21					
	4±1˚C		5±1˚C		6±1˚C		4±1˚C		5±1˚C		6±1˚C		4±1˚C		5±1˚C		6±1˚C		4±1˚C		5±1˚C		6±1˚C		4±1˚C		5±1˚C		6±1˚C	
	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D	H	D
24h	90.92± 0.05	7.06± 0.02	90.88± 0.25	7.08± 0.05	90.83± 0.14	7.13± 0.03	90.9±0 .14	8±0.0 1	90.79± 0.17	8.05± 0.04	90.68± 0.04	8.1±0. 01	90.85± 0.2	8.96± 0.01	90.32± 0.06	9.02± 0.31	89.79± 0.189	9.08± 0.21	89.54± 0.61	3.96± 0.09	89.58± 0.35	3.95± 0.15	89.63± 0.8	3.96± 0.05	55.05± 0.11	1.96± 0.01	53.15± 0.43	2.08± 0.01	50.1±0 .49	2.03± 0.06
48h	90.65± 0.41	7.16± 0.07	90.66± 0.04	7.22± 0.33	90.7±0 .64	7.23± 0.26	90.56± 0.96	8.13± 0.05	90.56± 0.55	7.85± 0.19	90.56± 0.18	7.93± 0.09	89.69± 0.24	9.16± 0.04	89.64± 0.44	9.17± 0.15	89.58± 0.55	9.14± 0.01	89.66± 0.55	3.1±0. 06	89.45± 0.22	3.28± 0.07	89 .39±0. 29	3.96± 0.01	49.8±0 .59	0.64± 0.05	28.18± 0.15	0.69± 0.04	15.16± 0.21	0.63± 0.01
72h	90.63± 0.09	7.1±0. 05	90.6±0 .23	7.09± 0.09	90.58± 0.85	7.03± 0.25	90.54± 0.98	7.99± 0.02	90.53± 0.55	8.01± 0.41	90.50± 0.11	8.06± 0.16	89.36± 0.47	8.14± 0.01	89.14± 0.09	8.18± 0.0 7	88.93± 0.26	8.13± 0.1	88.76± 0.76	3.13± 0.05	88.69± 0.03	3.08± 0.04	88.63± 0.59	3.03± 0.05	39.93± 0.52	0.34± 0.06	22.07± 0.7	0.37± 0.15	12.1±0 .07	0.35± 0.02
96h	90.54± 0.35	6.32± 0.11	90.48± 0.15	6.37± 0.01	90.4±0 .41	6.4±0. 15	80.16± 0.23	6.5±0. 33	77.51± 0.37	6.45± 0.05	73.36± 0.45	6.40± 0.01	59.94± 0.37	7.00± 0.41	59.43± 0.06	6.46± 0.16	46.93± 0.53	6.43± 0.17	19.93± 0.6	3.16± 0.07	19.51± 0.02	3.09± 0.08	19.46± 0.44	3.03± 0.07	0	0	0	0	0	0

It was observed that up to 15 days preservation early embryonic stages showed good hatching percentage up to 15 days, but the days delayed regarding hatching was very short (nearly 3 days). More over 96 hours embryo showed good hatching percentage when preserved for three days, but the days delayed was nearly one day short than up to 72 hours. So it can be concluded that up to ten days of preservation were found suitable for hatching percentage to ensure desired quantity of seed when required up to a delay of 9 days (Table 40). Regression analysis also confirmed present findings (Plate 15 and 16)

So it can be concluded that early embryos up to 72 hours can be preserved for up to 10 days at any cold shock with 4±1˚C to 6±1˚C, so that the differentiation period can be delayed to ensure seed supply at desired quantity when required. More over performances of 96 hour embryo can also meet the demand of seed supply to some extent.

4.5 Biochemical changes in embryonic developmental events of *A. assama*

4.5.1 Biochemical changes in embryonic developmental events during normal differentiation of *A. assama*

4.5.1.1 Biochemical changes in carbohydrate content in embryonic developmental events during normal differentiation of*A. assama*

ANOVA analysis confirmed that the changes in carbohydrate content during embryonic development are significant ($P<0.05$). Carbohydrate content was decreased from 24 hr (33.98±0.009) to 72 hr (25.76±0.005) old egg significantly, then increased on 96 hr (27.64±0.019) old and again decreased significantly till before hatching after 168 hr (13.32±0.009). (Table 41)

Table 41. Biochemical changes in carbohydrate content in embryonic developmental events during normal differentiation of *A. assama*

Embryo(hr)	Carbohydrate (mg/g)
24hr	33.98±0.009
48 hr	29.56±0.004
72hr	25.76±0.005
96hr	27.64±0.019
120hr	25.16±0.19
144hr	23.26±0.006
168hr	13.32±0.009
CD at 5%	1.63

4.5.1.2. Biochemical changes in protein content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in protein content during embryonic development are non significant (P>0.05). Protein content was decreased from 24 hr (163.74±0.035) to 72 hr (132.53±0.02) significantly and from 72 hr (132.53±0.02) to 96 hr (127.25 ±0.025) non significantly and then to 120 hr (121.17±0.04) old eggs, significantly. Again, it was increased on 144 hr (175.08±0.03) old eggs significantly and then decreased significantly till before hatching after 168 hr (112.17±0.058). (Table 42)

Table 42. Biochemical changes in protein content in embryonic developmental events during normal differentiation of *A. assam*

Embryo (hr)	Protein (mg/g)
24hr	163.74±0.035
48 hr	142.63±0.015
72hr	132.53±0.02
96hr	127.25 ±0.025
120hr	121.17±0.04
144hr	175.08±0.03
168hr	112.17±0.058
CD at 5%	5.836

4.5.1.3 Biochemical changes in cholesterol content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in cholesterol content during embryonic development are significant (P<0.05). Cholesterol content was increased from 24 hr (105.88±0.027) to 48 hr (111.76±0.021) old egg non significantly, then increased up to 96 hr (253.56±0.86) old significantly and again decreased non significantly till before hatching after 168 hr (108.82±0.02). (Table 43)

Table 43. Biochemical changes in cholesterol content in embryonic developmental events during normal differentiation of *A. assama*

Embryo (hr)	Cholesterol (mg%)
24hr	105.88±0.027
48 hr	111.76±0.021
72hr	188.23±0.009
96hr	253.56±0.86
120hr	200.61±0.019
144hr	129.76±0.02
168hr	108.82±0.02
CD at 5%	64.64

4.5.1.4 Biochemical changes in DNA content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in DNA content during embryonic development are significant ($P<0.05$). DNA content was increased from 24 hr (0.55±0.02) to 120 hr (2.45±0.025) old egg significantly, then increased up to 144 hr (2.60±0.045) old non significantly and finally increased significantly till before hatching after 168 hr (3.05±0.025). (Table 44)

Table 44. Biochemical changes in DNA content in embryonic developmental events during normal differentiation of *A. assama*

Embryo(hr)	DNA (mg/ml)
24hr	0.55±0.02
48 hr	1.25±0.03
72hr	1.95±0.015
96hr	2.15±0.035
120hr	2.45±0.025
144hr	2.60±0.045
168hr	3.05±0.025
CD at 5%	0.217

4.5.1.5 Biochemical changes in Threhalose content in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in Trehalose content during embryonic development are significant (P<0.05). Trehalose content was initially decreased from 24 hr (2.32±0.0125) to 48 hr (0.78±0.0118) old egg significantly, then increased up to 120 hr (59.13±0.002) old significantly and again decreased significantly till before hatching after 168 hr (12.45±0.058). (Table 45)

Table 45. Biochemical changes in Threhalose content in embryonic developmental events during normal differentiation of *A. assama*

Embryo (hr)	Trehalose(mg/ml)
24hr	2.32±0.0125
48 hr	0.78±0.0118
72hr	14.78±0.008
96hr	17.12±0.005
120hr	59.13±0.002
144hr	16.34±0.017
168hr	12.45±0.058
CD at 5%	4.996

4.5.1.6 Biochemical changes in NAD-SDH in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in NAD-SDH content during embryonic development are significant (P<0.05). NAD-SDH content was initially increased from 24 hr (0.19±0.004) to 72 hr (3.13 ±0.021) old egg significantly, then decreased up to 120 hr (0.42 ±0.015) old significantly and again increased significantly by 144 hr (2.04±0.001) and finally decreased by 168 hr (1.98± 0.01). (Table 46)

Table 46. Biochemical changes in NAD-SDH in embryonic developmental events during normal differentiation of *A. assama*

Embryo(Hr)	NAD-SDH(Ux10^{-3}/ml)
24hr	0.19±0.004
48 hr	1.92±0.055
72hr	3.13 ±0.021
96hr	2.73 ±0.025
120hr	0.42 ±0.015
144hr	2.04±0.001
168hr	1.98± 0.01
CD at 5%	0.279

4.5.1.7 Biochemical changes in NADPH-Peroxidase in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in NADPH-Peroxidase content during embryonic development are non significant (P>0.05). NADPH-Peroxidasecontent was initially increased from 24 hr (0.0395 ± 0.0005) to 48 hr (0.0450±0.0001) old egg significantly, then decreased up to 96 hours egg (0.0142±0.0002) significantly and again increased in 120 hr (0.0517±0.0674) old significantly and then decreased non significantly till before hatching after 168 hr (0.0244±0.0004). (Table 47)

Table 47. Biochemical changes in NADPH-Peroxidase in embryonic developmental events during normal differentiation of *A. assama*

Embryo age (h)	Peroxidase in x10^{-3} U/mg
24h	0.0395 ± 0.0005
48h	0.0450±0.0001
72h	0.0335±0.0004
96h	0.0142±0.0002
120h	0.0517±0.0674
144h	0.0487±0.0002
168h	0.0244±0.0004
CD at 5%	0.0035

4.5.1.8 Biochemical changes in XO in embryonic developmental events during normal differentiation of *A. assama*

ANOVA analysis confirmed that the changes in XO content during embryonic development are significant ($P<0.05$). XO content was initially increased from 24 hr (0.059±0.003) to 48 hr (0.197±0.002) old egg significantly, then increased up to 120 hr (1.102±0.004) old significantly and decreased non significantly (1.219±0.009) but again increased before hatching after 168 hr (1.552±0.019). (Table 48)

Table 48. Biochemical changes in XO in embryonic developmental events during normal differentiation of *A. assama*

Embryo age	XO in x10-3U/ml
24h	0.059±0.003
48h	0.197±0.002
72h	0.118±0.004
96h	0.373±0.009
120h	1.102±0.004
144h	1.219±0.009
168h	1.552±0.019
CD at 5%	0.156

4.5.2 Biochemical changes in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.1 Biochemical changes in carbohydrate content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.1.1 Biochemical changes in carbohydrate content in different identified embryonic stages

24 Hours

In case of 24 hr eggs there was significant decrease in carbohydrate level after 3 (35.81±0.05), 7 (20.27±0.09) and 10 (6.25±0.03) days preservation. There was

significant increase of carbohydrate level than control after 3days (35.81±0.05) preservation and significant decrease after 7 (20.27±0.09) and 10 days (6.25±0.03) preservation. (Table 49)

48 Hours

In case of 48 hr eggs there was significant decrease in carbohydrate level after 3(27.28±0.06), 7 (13.72±0.11) and 10 days (12.45±0.17) preservation. There was significant decrease of carbohydrate level than control after 3 (27.28±0.06), 7 (13.72±0.11) and 10 days (12.45±0.17) preservation. (Table 49)

72 Hours

In case of 72 hr eggs there was significant decrease in carbohydrate level after 3 (10.88±0.15), 7 (12.46±0.13) and 10 days (12.45±0.05) preservation. There was significant decrease after 3 (10.88±0.15), 7 (12.46±0.13) and 10 days (12.45±0.05) preservation. (Table 49)

96 Hours

In case of 96 hr eggs there was non significant increase of carbohydrate level between 3 (21.8±0.05) and 7 days (20.26±0.07) preservation but significant decrease was reported after 10 days (14.8±0.02) preservation. There was significant decrease of carbohydrate level than control after 3 (21.8±0.05), 7 (20.26±0.07) and 10 days (14.8±0.02) preservation. (Table 49)

4.5.2.1.2 Biochemical changes in carbohydrate content in different identified embryonic stages at different cold preservation periods

After 3Days

After 3 days preservation, carbohydrate level decreased in 48 hr (27.28±0.06), 72 hr (10.88±0.15) and 96 hr (21.8±0.05) than 24 hr (35.81±0.05) level significantly. (Table 49)

After 7 Days

After 7 days preservation, carbohydrate level decreased significantly in 48 (13.72±0.11) and 72 hr (12.46±0.13) and non significantly increased in 96 hr (20.26±0.07) than 24 hr (20.27±0.09) . (Tabl 49)

After 10 Days

After 10 days preservation, carbohydrate level increased in 48 (12.45±0.17), 72(12.45±0.05) and 96 hr (14.8±0.02) significantly, than 24 hr (6.25±0.03). (Table 49)

But at normal development carbohydrate level decreased significantly by 48 hr (29.56±0.04), 72 hr (25.76±0.05) and non significantly increased in 96 hr (27.64±0.19). (Table 49)

Table 49. Biochemicalchanges in carbohydrate content (mg/g) in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

Preservation Days	24h	48h	72h	96h
3	35.81±0.05	27.28±0.06	10.88±0.15	21.8±0.05
7	20.27±0.09	13.72±0.11	12.46±0.13	20.26±0.07
10	6.25±0.03	12.45±0.17	12.45±0.05	14.8±0.02
Control	33.98±0.09	29.56±0.04	25.76±0.05	27.64±0.19
CD at 5%	2.02			

4.5.2.2 Biochemical changes in protein content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.2.1 Biochemical changes in protein content in different identified embryonic stages

24 Hours

In case of 24 hr eggs there was significant increase in protein level after 7 (265.63±0.21) days and 10 days (253.52±0.04) preservation than after 3 days

(155.65±0.05) preservation. There was significant increase of protein level than control after 7 (265.63±0.21) and 10 days (253.52±0.04) preservation, but non significant decrease after 3days (155.65±0.05) preservation. (Table 50)

48 Hours

In case of 48 hr eggs there was significant increase in protein level after 7 (280.05±0.31) and 10 days (233.5±0.11) preservation than 3 days (154.68±0.06) preservation. There was significant increase of protein level than control after 7 (280.05±0.31) and 10 days (233.5±0.11) preservation and non significant increase was recorded after 3 days (154.68±0.06) preservation. (Table 50)

72 Hours

In case of 72 hr eggs there was significant increase in protein level after 7 (273.52±0.07) and 10 days (248.29±0.06) preservation than 3 days (155.2±0.11) preservation. There was significant increase of protein level than control after 7 (273.52±0.07) and 10 days (248.29±0.06) preservation and non significant increase was recorded after 3 days (155.2±0.11) preservation. (Table 50)

96 Hours

In case of 96 hr eggs there was significant increase in protein level after 7 (242.52±0.07) and 10 days (237.8±0.14) preservation than 3 days (154.63±0.05) preservation. There was significant increase of protein level than control after 3 (154.63±0.05), 7 (242.52±0.07) and 10 days (237.8±0.14) preservation. (Table 50)

4.5.2.2.2Biochemical changes in protein content in different identified embryonic stages at different cold preservation periods

After 3 Days

After 3 days preservation, non significant change in protein level was evident in 48 (154.68±0.06), 72 (155.2±0.11) and 96 hr (154.63±0.05) egg than 24 hr (155.65± 0.05) egg. (Table 50)

After 7 Days

After 7 days preservation, non significant change in protein level was evident in 48 (280.05±0.31), 72 (273.52±0.07) and 96 hr (242.52±0.07) egg than 24 hr (265.63±0.21) egg. (Table 50)

After 10 Days

After 10 days preservation, non significant change in protein level was evident in 48 (233.5±0.11), 72 (248.29±0.06) and 96 hr (237.8±0.14) egg than 24 hr (253.52±0.04) egg. (Table50)

But at normal development protein level increased non significantly from 24 hr (163.74±0.035), 48 hr (142.6±0.015), 72 hr (132.53±0.02) and 96 hr (127.25±0.025). (Table 50)

Table 50. Biochemical changes in protein content (mg/g) in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

Preservation Days	24h	48h	72h	96h
3	155.65±0.05	154.68±0.06	155.2±0.11	154.63±0.05
7	265.63±0.21	280.05±0.31	273.52±0.07	242.52±0.07
10	253.52±0.04	233.5±0.11	248.29±0.06	237.8±0.14
Control	163.74±0.035	142.6±0.015	132.53±0.02	127.25±0.025
CD at 5%	30.427			

4.5.2.3 Biochemical changes in cholesterol content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.3.1 Biochemical changes in cholesterol content in different identified embryonic stages

24 Hours

In case of 24 hr eggs there was non significant decrease in cholesterol level after 3(75.90±0.05) and 7 (73.19±0.10) and 10 days (64.33±0.05) preservation. There was significant decrease of cholesterol level than control after 3(75.90±0.05) , 7 (73.19±0.10)and 10 days (64.33±0.05) preservation. (Table 51)

48 Hours

In case of 48 hr eggs there was no significant decrease in cholesterol level after 3(76.57±0.07), 7(71.75±0.04) and 10 days (60.82±0.08) preservation. There was significant decrease of cholesterol level than control after 3 (76.57±0.07), 7 (71.75±0.04) and 10 days (60.82±0.08) preservation. (Table 51)

72 Hours

In case of 72 hr eggs there was no significant decrease in cholesterol level after 3(70.12±0.08), 7 (69.03±0.02) and 10 days (59.07±0.03) preservation. There was significant decrease of cholesterol level than control after 3(70.12±0.08), 7(69.03±0.02) and 10 days (59.07±0.03) preservation. (Table 51)

96 Hours

In case of 96 hr eggs there was no significant decrease in cholesterol level after 3 (72.68±0.06), 7 (65.75±0.04) and 10 days (57.01±0.09) preservation. There was significant decrease of cholesterol level than control after 3 (72.68±0.06), 7(65.75±0.04) and 10 days (57.01±0.09) preservation. (Table 51)

4.5.2.3.2Biochemical changes in cholesterol content in different identified embryonic stages at different cold preservation periods

After 3 Days

After 3 days preservation, non significant change in cholesterol level was evident in 48 (76.57±0.07), 72(70.12±0.08) and 96 hr (72.68±0.06) egg than 24 hr (75.90±0.05) egg. (Table 51)

After 7 Days

After 7 days preservation, non significant change in cholesterol level was evident in 48 (71.75±0.04), 72 (69.03±0.02) and 96 hr (65.75±0.04) egg than 24 hr (73.19±0.10) egg. (Table 51)

After 10 Day

After 10 days preservation, non significant change in protein level was evident in 48 (60.82±0.08), 72(59.07±0.03) and 96 hr (57.01±0.09) egg than 24 hr (64.33±0.05) egg. (Table 51)

But during normal development, cholesterol level increased non significantly from 24 hr (105.88±0.028) to 48 hr (111.76±0.022), but significantly by 72 hr (188.23±0.010) and 96 hr (253.56±0.87). (Table 51)

Table 51. Biochemical changes in cholesterol content (mg %) in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

Preservation Days	24h	48h	72h	96h
3	75.90±0.05	76.57±0.07	70.12±0.08	72.68±0.06
7	73.19±0.10	71.75±0.04	69.03±0.02	65.75±0.04
10	64.33±0.05	60.82±0.08	59.07±0.03	57.01±0.09
Control	105.88±0.028	111.76±0.022	188.23±0.010	253.56±0.87
CD at 5%	9.08			

4.5.2.4 Biochemical changes in DNA content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.4.1 Biochemical changes in DNA content in different identified embryonic stages

24 Hours

In case of 24 hr eggs there was significant increase in DNA level after 7 (1.97±0.08) and 10 days (3.01±0.05) preservation than 3 days (0.051±0.01) preservation. There was significant decrease of DNA level than control after 3 days (0.051±0.01) preservation, but significantly increased after 7 (1.97±0.08) and 10 (3.01±0.05) days preservation. (Table 52)

48 Hours

In case of 48 hr eggs there was significant increase in DNA level after 7 (2.17±0.09) and 10 days (3.01±0.05) preservation than 3 days (0.073±0.03) preservation. There was significant decrease of DNA level than control after 3 days (0.073±0.03) preservation, but significantly increased after 7 (2.17±0.09) and 10 days (3.01±0.05) preservation. (Table 52)

72 Hours

In case of 72 hr eggs there was significant increase in DNA level after 7 (2.44±0.09) and 10 days (3.18±0.17) preservation than 3days (0.099±0.07) preservation. There was significant decrease of DNA level than control after 3 days (0.099±0.07) preservation, but significantly increased after 7 (2.44±0.09) and 10 days (3.18±0.17) preservation. (Table 52)

96 Hours

In case of 96 hr eggs there was significant increase in DNA level after 7 (2.61±0.02) and 10 days (3.26±0.06) preservation than 3 days (0.127±0.01) preservation. There was significant decrease of DNA level than control after 3days (0.127±0.01) preservation, but significantly increased after 7 (2.61±0.02) and 10 days (3.26±0.06) preservation. (Table 52)

4.5.2.4.2 Biochemical changes in DNA content in different identified embryonic stages at different cold preservation periods

After 3Days

After 3 days preservation, non significant change in DNA level was evident in 48 (0.073±0.03), 72 (0.099±0.07) and 96 hr (0.127±0.01) egg than 24 hr (0.051±0.01) eggs. (Table 52)

After 7 Days

After 7 days preservation, non significant change in DNA level was evident between 24 hr (1.97±0.08) and 48 hr (2.17±0.09) old eggs, but significant increase was noticed between 72(2.44±0.09) and 96 hr (2.61±0.02) eggs. (Table 52)

After 10 Days

After 10 days preservation, non significant change in DNA level was evident in 48(3.17±0.21), 72 (3.18±0.17) and 96 hr (3.26±0.06) egg than 24 hr (3.01±0.05) egg. (Table 52)

But during normal development, DNA level was increased significantly from 24 (0.55±0.02) hr to 48(1.25±0.03) hr and 72 (1.95±0.015) hr but non significantly between 72(1.95±0.015) hr and 96 (2.15±0.35) hr. (Table 52)

Table 52. Biochemical changes in DNA content (mg/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

Preservation Days	24h	48h	72h	96h
3	0.051±0.01	0.073±0.03	0.099±0.07	0.127±0.01
7	1.97±0.08	2.17±0.09	2.44±0.09	2.61±0.02
10	3.01±0.05	3.17±0.21	3.18±0.17	3.26±0.06
Control	0.55±0.02	1.25±0.03	1.95±0.015	2.15±0.35
CD at 5%	0.328			

4.5.2.5 Biochemical changes in trehalose content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.5.1 Biochemical changes in trehalose content in different identified embryonic stages

24 Hours

In case of 24 hr eggs, there was significant decrease, in trehalose level after 3 (35.77±0.04), 7 (6.25±0.05) and 10 days (20.24±0.06) preservation. There was significant increase of trehalose level than control after 3 (35.77±0.04) and 10 days (20.24±0.06) preservation and non significantly changed after 7days (6.25±0.05) preservation. (Table 53)

48 Hours

In case of 48 hr eggs there was significant decrease in trehalose level after 7(12.43±0.02) and 10 days (17.72±0.04) preservation than 3 days (28.78±0.04) preservation. There was significant increase of trehalose level than control after 3 (28.78±0.04), 7 (12.43±0.02) and 10 days (17.72±0.04) preservation. (Table 53)

72 Hours

In case of 72 hr eggs there was non significant increase in trehalose level after 10 days (16.04±0.08) preservation than 3 days (10.82±0.01) preservation but significant increase was reported after 7 days (53.68±0.05) preservation. There was significant increase of trehalose level than control after 7 days (53.68±0.05) preservation and non significantly changed after 3 (10.82±0.01) and 10days (16.04±0.08) preservation. (Table 53)

96 Hours

In case of 96 hr eggs there was non significant change in trehalose level after 3 (20.25±0.04), 7 (21.74±0.04) and 10 days (14.78±0.04) preservation. There was non significant increase of trehalose level than control after 3 (20.25±0.04), 7 (21.74±0.04) and 10days (14.78±0.04) preservation. (Table 53)

4.5.2.5.2Biochemical changes in trehalose content in different identified embryonic stages at different cold preservation periods

After 3Days

After 3 days preservation, significant change in trehalose level was evident in 48 (28.78±0.04), 72 (10.82±0.01) and 96 hr (20.25±0.04) egg than 24 hr (35.77±0.04) eggs. (Table 53)

After 7 Days

After 7 days preservation, significant change in trehalose level was evident in 48 (12.43±0.02), 72 (53.68±0.05) and 96 hr (21.74±0.04) egg than 24 hr (6.25±0.05) eggs. (Table 53)

After 10 Days

After 10 days preservation, non significant change in trehalose level was evident in 48 (17.72±0.04), 72 (16.04±0.08) and 96 hr (14.78±0.04) egg than 24 hr (20.24±0.06) eggs. (Table 55)

But during normal development, trehalose level was decreased non significantly from 24 hr (0.778±0.02) to 48hr (0.778±0.02), but significantly increased from 48 hr (0.778±0.02) to 72 hr (14.782±0.02) and 96 hr (17.12±0.006). (Table 53)

Table 53. Biochemical Changes in trehalose (mg/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

Preservation Days	24h	48h	72h	96h
3	35.77±0.04	28.78±0.04	10.82±0.01	20.25±0.04
7	6.25±0.05	12.43±0.02	53.68±0.05	21.74±0.04
10	20.24±0.06	17.72±0.04	16.04±0.08	14.78±0.04
Control	2.34±0.005	0.778±0.02	14.782±0.02	17.12±0.006
CD at 5%	6.345			

4.5.2.6 Biochemical changes in NAD-SDH content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.26.1 Biochemical changes in NAD-SDH content in different identified embryonic stages

24 Hours

In case of 24 hr eggs there was no significant increase in NAD-SDH level after 3(2.53±0.05) and 7 days (2.6±0.11) preservation but significantly decreased after 10 days (0.692±0.05) preservation. There was significant increase of NAD-SDH level than control after 3(2.53±0.05), 7(2.6±0.11) and 10 days (0.692±0.05) preservation. (Table 54)

48 Hours

In case of 48 hr eggs there was no significant increase in NAD-SDH level after 3(2.05±0.06) and 7 days (2.18±0.07) preservation but significantly decreased after 10 days (1.05±0.02) preservation. There was significant increase of NAD-SDH level than control after 3(2.05±0.06), 7(2.18±0.07) and 10 days (1.05±0.02) preservation. (Table 54)

72 Hours

In case of 72 hr eggs there was significant increase in NAD-SDH level after 3 (3.06±0.09) and 7 days (5.26±0.03) preservation but significantly decreased after 10 days (1.37±0.02) preservation. There was significant increase of NAD-SDH level than control after 3 (3.06±0.09), 7 (5.26±0.03) and 10 days (1.37±0.02) preservation. (Table 54)

96 Hours

In case of 96 hr eggs there was no significant increase in NAD-SDH level after 3 (0.87±0.14) and 7 days (2.88±0.03) preservation but significantly decreased after 10 days (3.36±0.02) preservation. There was significant increase of NAD-SDH level than control after 3 (0.87±0.14) , 7 (2.88±0.03) and 10 days (3.36±0.02) preservation. (Table 54)

4.5.2.6.2Biochemical changes in NAD-SDH content in different identified embryonic stages at different cold preservation periods

After 3Days

After 3 days preservation, NAD-SDH level decreased in 48 hr (2.05±0.06) , non significantly, then increased by 72 hr (3.06±0.09) significantly and also decreased by 96 hr (0.87±0.14) significantly. (Table 54)

After 7 Days

After 7 days preservation, NAD-SDH level decreased in 48 hr (2.18±0.07), non significantly, then increased by 72 hr (5.26±0.03) old significantly and also decreased by 96 hr (2.88±0.03) significantly. (Table 54)

After 10 Day

After 10 days preservation, NAD-SDH level decreased in 48 hr (1.05±0.02) non significantly, then increased by 72 hr (1.37±0.02) significantly and also decreased by 96 hr (3.36±0.02) significantly. (Table 54)

But during normal development, NAD-SDH level increased significantly by 24hr (0.19±0.004), 48 hr (1.03±0.04) , 72 hr (1.39±0.001) and 96 hr (3.32±0.002) . (Table 54)

Table 54. Biochemical changes in NAD-SDH content (Ux10^{-3}/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

Preservation Days	24h	48h	72h	96h
3	2.53±0.05	2.05±0.06	3.06±0.09	0.87±0.14
7	2.6±0.11	2.18±0.07	5.26±0.03	2.88±0.03
10	0.692±0.05	1.05±0.02	1.37±0.02	3.36±0.02
Control	0.19±0.004	1.03±0.04	1.39±0.001	3.32±0.002
CD at 5%	0.219			

4.5.2.7 Biochemical changes in NADPH-Peroxidase content in different identified embryonic stages at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.7.1 Biochemical changes in NADPH-Peroxidase content in different identified embryonic stages

24 Hours

In case of 24 hr eggs there was significant increase in NADPH-Peroxidase level after 7 days (0.100±0.04) preservation but, significantly decreased after 10 days (0.033±0.05) preservation than 3 days (0.0126±0.02) preservation. There was significant decrease of NADPH-Peroxidase level than control after 3 days (0.0126±0.02) preservation, but significantly increased after 7 days (0.100±0.04) preservation and non significantly increased after 10 days (0.033±0.05) preservation. (Table 55)

48 Hours

In case of 48 hr eggs there was significant decrease in NADPH-Peroxidase level after 7 days (0.059±0.005) preservation and significantly increased after 10 days (0.458±0.008) preservation than 3 days (0.199±0.007) preservation. There was significant increase of NADPH-Peroxidase level than control after 3 days (0.199±0.007) preservation, but non significantly decreased after 7 days (0.059±0.005) preservation and significantly increased after 10 days (0.458±0.008) preservation. (Table 55)

72 Hours

In case of 72 hr eggs there was significant increase in NADPH-Peroxidase level after 7 days (0.1003±0.002) preservation but, significantly decreased after 10 days (0.062±0.005) preservation than 3 days (0.018±0.007) preservation. There was significant decrease of NADPH-Peroxidase level than control after 3 days (0.018±0.007) preservation, but significantly increased after 7 days (0.1003±0.002) and 10 days (0.062±0.005) preservation. (Table 55)

96 Hours

In case of 96 hr eggs there was significant decrease in NADPH-Peroxidase level after 7 days (0.085±0.004) preservation and significantly increased after 10 days (0.212±0.03) preservation than 3 days (0.184±0.003) preservation. There was significant increase of

NADPH-Peroxidase level than control after 3(0.184±0.003), 7(0.085±0.004) and 10 days (0.212±0.03) preservation. (Table 55)

4.5.2.7.2 Biochemical changes in NADPH-Peroxidase content in different identified embryonic stages at different cold preservation periods

After 3Days

After 3 days preservation, NADPH-Peroxidase level increased by 48 hr (0.199±0.007) significantly, then decreased by 72 hr (0.018±0.007) significantly and also increased by 96 hr (0.184±0.003) significantly. (Table 55)

After 7 Days

After 7 days preservation, NADPH-Peroxidase level decreased in 48 hr (0.059±0.005) , significantly, then increased by 72 (0.1003±0.002) hr old significantly and also decreased by 96 hr (0.085±0.004) significantly. (Table 55)

After 10 Day

After 10 days preservation, NADPH-Peroxidase level increased in 48 hr (0.458±0.008) significantly, then decreased by 72 hr (0.062±0.005) significantly and also increased by 96 hr (0.212±0.03) significantly. (Table 55)

But during normal development, NADPH-Peroxidase level maintained non significant variation by 24 hr (0.04±0.05),48 hr (0.045±0.04) and 72 hr (0.034±0.04) but decreased significantly by 96 hr (0.014±0.04). (Table 55)

Table 55. Biochemical changes in NADPH-Peroxidase content (Ux10^{-3}/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*.

Preservation Days	24h	48h	72h	96h
3	0.0126±0.02	0.199±0.007	0.018±0.007	0.184±0.003
7	0.100±0.04	0.059±0.005	0.1003±0.002	0.085±0.004
10	0.033±0.05	0.458±0.008	0.062±0.005	0.212±0.03
Control	0.04±0.05	0.045±0.04	0.034±0.04	0.014±0.04
CD at 5%	0.019			

4.5.2.8Biochemical changes in XO content in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

4.5.2.1 Biochemical changes in XO content in different identified embryonic stage

24 Hours

In case of 24 hr eggs there was significant decrease in XO level after 7 days (0.39±0.09) preservation and non significantly increased after 10 days (0.42±0.01) preservation than 3 days (0.55±0.03) preservation. There was significant increase of XO level than control after 3 (0.55±0.03), 7(0.39±0.09) and 10 days (0.42±0.01) preservation. (Table 56)

48 Hours

In case of 48 hr eggs there was significant decrease in XO level after 7 days (0.28±0.01) preservation, but significantly increased after 10 days (0.39±0.01) preservation than 3 days (0.52±0.07) preservation. There was significant increase of XO level than control after 3(0.52±0.07),7 (0.28±0.01) and 10 days (0.39±0.01) preservation. (Table 56)

72 Hours

In case of 72 hr eggs there was significant decrease in XO level after 7 days (0.14±0.02) preservation and significantly increased after 10 days (0.35±0.05) preservation than 3 days(1.21±0.05) preservation. There was significant increase of XO level than control after 3 (1.21±0.05), 7 (0.14±0.02) and 10 days (0.35±0.05) preservation. (Table 56)

96 Hours

In case of 96 hr eggs there was significant increase in XO level after 7 (0.47±0.03) and 10 days (0.67±0.05) preservation than 3 days (0.22±0.04) preservation. There was significant decrease of XO level than control after 3 days (0.22±0.04) but increased after 7(0.47±0.03) and 10 days (0.67±0.05) preservation significantly. (Table 56)

4.5.2.2Biochemical changes in XO **content in different identified embryonic stage at different cold preservation periods**

After 3Days

After 3 days preservation, XO level decreased by 48 hr (0.52±0.07) non significantly, then increased by 72 hr (1.21±0.05) significantly and also decreased by 96 hr (0.22±0.04) significantly. (Table 56)

After 7 Days

After 7 days preservation, XO level decreased by 48 (0.28±0.01) and 72 hr (0.14±0.02) significantly and then increased by 96 hr (0.47±0.03) significantly. (Table 56)

After 10 Day

After 10 days preservation, XO level decreased by 48 hr (0.39±0.01) non significantly, then decreased by 72 hr (0.35±0.05) significantly and also increased by 96 hr (0.67±0.05) significantly. (Table 56)

But during normal development, XO level significantly increased by 24 hr (0.06±0.02) and 48 hr (0.20±0.01), then decreased by 72 hr (0.12±0.01) but again increased significantly by 96 hr (0.37±0.08). (Table 56)

Table 56. Biochemical changes in XO content ($Ux10^{-3}$/ml) in different identified embryonic stage at different cold preservation periods during low temperature stress of *A. assama*

Preservation Days	24h	48h	72h	96h
3	0.55±0.03	0.52±0.07	1.21±0.05	0.22±0.04
7	0.39±0.09	0.28±0.01	0.14±0.02	0.47±0.03
10	0.42±0.01	0.39±0.01	0.35±0.05	0.67±0.05
Control	0.06±0.02	0.20±0.01	0.12±0.01	0.37±0.08
CD at 5%	0.046			