# FREEZABILITY, ACROSOME STATUS AND CONCEPTION RATE OF FROZEN BULL SPERMATOZOA AT DIFFERENT AGES SUPPLEMENTED WITH CAFFEINE

**Shitta, A. A.**
**Animal Production Research Institute, Dokki, Giza, Egypt.**

## ABSTRACT

Semen was collected from five of each of young (3-5 years) and old (6-10 years) Friesian bulls and extended with tris-yolk fructose extender (1 semen: 20 extender). The extended semen was cooled to 5ºC over 2 hours, then frozen in straws. After 24 hours, the frozen semen was thawed and supplemented with caffeine at levels of 0 and 10 mM/100 ml. Frozen-thawed semen with caffeine was then incubated at 37ºC for 4 hours. The percentages of post-thawing motility, freezability and acrosome status of spermatozoa, were estimated. Conception rates for the thawed-semen frozen in each of young and old bulls, were also assessed.

The results showed that, freezing of the young bull semen maintained ($P< 0.01$) higher the percentages of post-thawing motility and freezability than old bull spermatozoa. Supplementation of caffeine at a level of 10 mM to the thawed-frozen semen in each of young or old bull significantly ($P< 0.01$) higher the percentages of post-thawing motility and freezability of spermatozoa, during thawing-incubation at 37ºC for up to 4 hours. The incubation time had a significant ($P<0.01$) effect on decreasing the percentages of post-thawing motility and freezability of young and old bull spermatozoa. The percentages of live spermatozoa with intact acrosome decreased ($P<0.01$) and the percentages of dead spermatozoa with intact acrosome increased ($P<0.01$), however, the percentages of live and dead spermatozoa with detached acrosomes showed decreased significantly ($P<0.01$) with the successive time of incubation. The percentages of live spermatozoa with each of intact or detached acrosomes in young were insignificantly higher than old bulls. However, the percentages of dead spermatozoa with each of intact or detached acrosomes in young were significantly ($P<0.01$) lower than old bulls. Supplementation of caffeine at a level of 10 mM to the thawed-frozen semen showed insignificant higher the percentage of live spermatozoa with intact or detached acrosomes and significantly($P<0.01$) lower the percentages of dead spermatozoa with intact or detached acrosomes than free-caffeine medium.

Conception rates for cows artificially inseminated with the thawed-frozen semen were insignificantly higher in young than old bulls.

**Keywords**: Bull semen, freezability, acrosome status, conception rate, caffeine

## INTRODUCTION

Artificial insemination (AI) is now widely used in cattle production systems within European countries. However, AI in Egypt is still practised on a very limited scale in all cattle farms. One of the main constraints is the processing and storage of bull semen for prolonged periods, while still preserving acceptable fertility. On the other hand, success of artificial

insemination is dependent on the quality of semen obtained and its capacity for dilution and storage with minimum loss of fertilizing ability.

Various additives have been incorporated into semen extenders to enhance sperm longevity and fertility (Maule, 1962). The addition of phosphodiesterase inhibitors which prevent the breakdown of cyclic 3, 5 adenosine monophosphate (cAMP) such as caffeine, markedly increased respiration and maintained motility of bovine spermatozoa (Garbers *et al*., 1971; Simpson and White, 1987 and Zeidan, 1994). On the other hand, there are wide discrepancies between the several studies of the semen quality due to many interfering factors. The main factors are, species, breeds within species, age of the bull and seasonal environmental circumastances (climate and nutrition). However, attention has not been focoused on the effect of bull age on freezability and acrosomal status of spermatozoa added with caffeine which may help in understanding and improving the reproductive efficiency in the cattle which is still somewhat masked.

The present study aimed to investigate the effects of age of bull (young or old of age) on post-thawing motility, freezability and acrosome status of spermatozoa supplemented with caffeine. Conception rate as affected by age of bulls was also assessed.

## MATERIALS AND METHODS

The present study was carried out in Animal Production Research Station, Sakha,Kafr El-Sheikh Province, located in the north eastern part of the Nile Delta, Animal Production Research Institute, Egypt.

Twelve Friesian bulls were allocated according to their ages to young (3-5 years) and old (6-10 years). Semen was collected twice weekly over a five weeks interval from bulls with aid of an artificial vagina. Immediately after collection, semen was evaluated and only ejaculates showing active wave motion (<70%) were pooled. The pooled semen was extended with tris-yolk fructose extender by the two steps method as described by Colas (1975). The final extension rate was 1 semen: 20 extender. The extended semen was cooled to 5ºC in a refrigerator over 2 hours. Glycerol was added at a level of 7 % and was left at 5 ºC for 6 hours as equilibration period. Briefly, the cooled semen was then frozen in straws (0.25 ml). Straws were then placed in a rack approximately 4 cm above a liquid nitrogen vapiour (-75 ºC). After 10 minutes, the straws were immersed in a cryogenic refrigerator containing liquid nitrogen (-196 ºC). After 24 hours, holding the straws at the closed end and dipped in a water bath at 37 ºC for 30 seconds. The thawed-semen was then equally divided into two portions and supplemented with caffeine (Sigma Chemical Co., St. Lowis, MO., USA) at levels of 0 and 10 mM/100 ml. The thawed-semen supplemented with caffeine was then incubated at 37ºC for 4 hours in a water bath. The percentages of post-thawing motility and freezability of the young and old bull spermatozoa, were assessed by using a phase contrast microscope according to Patt and Nath (1969) and Zeidan (1994). Acrosome status was examined by the dual stain procedure as the method described by Didion *et al*. (1989). The following

categories were detected: a. live spermatozoa with intact acrosome (LIA), b. live spermatozoa with detached acrosome (LDA), c. dead spermatozoa with intact acrosome (DIA) and d. dead spermatozoa with detached acrosome (DDA).

In the fertility trial, 50 and 60 normally cyclic cows were artificially inseminated with the thawed of young and old bull semen, respectively. The number of motile spermatozoa per insemination was about 20 X $10^6$. Conception rate was estimated on the basis of pregnancy diagnosis by the rectal palpation after 60 days from date of insemination.

Data were statistically analyzed using least square analysis of variance according to Snedecor and Cochran (1982). Percentage values were transformed to arcsin values before being statistically analyzed. Duncan's multiple range test (Duncan, 1955) were used for the multiple comparisons. Conception rates were analyzed by Chi-square test.

## RESULTS AND DISCUSSION

**Post-thawing motility and freezability of spermatozoa**

Table 1 and 2 show that, the percentages of post-thawing motility and freezability of the young bulls were significantly ($P<0.01$) higher than old bulls spermatozoa, during incubation at 37ºC for 4 hours. Similar trends were reported by Almquist and Amann (1976), Everett and bean (1982) and Troconiz *et al*. (1991). The advancement of age revealed hypoactive Leydig cells which are considered to be testosterone producing factor so, this reflected on a bad semen quality produced by the aged animals (Tingari *et al*., 1993). Moreover, histological observations on bull tests revealed a progressive intertubular fibrosis, atrophic tubular changes and a reduction of siminiferous tubules surface to testis volume ratio with advancing age (Humphery and Ladds, 1975).

Supplementation of caffeine at a level of 10 mM to the thawed-frozen semen in each of young or old bulls significantly ($P<0.01$) higher the percentages of post-thawing motility and freezability of spermatozoa than free-caffeine medium, during incubation at 37ºC for 4 hours. Similarly, Zeidan (1994) found that addition of caffeine at a level of 10 mM to the thawed-bull semen increased significantly ($P<0.01$) the percentages of post-thawing motility and freezability of spermatozoa. Similar trend was reported by Schoenfeld *et al*. (1975) in human, Miyamoto and Nishikawa (1979) and El-Gaafary (1990) and Zeidan (1994) in Friesian bulls and El-Azab *et al*. (1998) in Buffalo bulls. These findings may be attributed to the methylxanthines group which acts as a phosphodiestrase inhibitor. The mechanism by which caffeine stimulates sperm motility is thought to involve the inhibition of phosphodiestrase enzyme responsible for breakdown of cyclic-adinosine monophosphate (cAMP) with consequent of accumulation of cyclic nucleotids especially cAMP within the sperm cells (Schoenfeld *et al*., 1975 and Tash and Means, 1983). Spermatozoal motility is partially controlled by cAMP and cyclic-guanosine monophosphate (cGMP). Glycolysis and tricarboxylic acid cycle was accelerated by cAMP with consequent increase in energy

production. It was evident that, cAMP stimulates sperm motility by direct action on the axoneme of the tail (Lindemann, 1978) or by indirectly action on the cell membrane as a secondary messenger (Garbers and Kopf, 1980).

1,2

The percentages of post-thawing motility and freezability of spermatozoa decreased significantly ($p<0.01$) as the time of incubation advanced. This may be due to the increase in lactic acid accumulation and that changes in pH of the media which induce the metabolic activity of spermatozoa and consequently, the sperm cell motility decrease (Zeidan *et al*., 1998).

**Acrosome status**

Data presented in Table 3 showed that, the percentages of live spermatozoa with intact or detached acrosomes in young bulls were significantly ($p<0.01$) higher than old bulls. However, the percentages of dead spermatozoa with intact or detached acrosomes in young bulls were significantly ($P<0.01$) lower than old bulls as shown in Table 4. Similarly, addition of caffeine at a level of 10 mM to the thawed-frozen young or old bulls semen showed insignificant higher the percentages of live and significantly ($P<0.01$) lower of dead spermatozoa with intact or detached acrosomes than free-caffeine medium. Lenz *et al*. (1977), Jones and Stewart (1979) and Zeidan *et al*. (1998) showed that subsequent freezing and thawing caused considerable ultrastructural changes to the acrosomes (disruption of the plasma and outer acrosomal membranes and dispersion of the acrosomal contents) and middle pieces (breakage of the plasma membrane and a reduction in the electron density of the mitochondrial matrix) of a high proportion of spermatozoa.

The percentages of live spermatozoa with intact acrosome decreased ($P<0.01$) and the percentages of dead spermatozoa with intact acrosome increased significantly ($P<0.01$), however, the percentages of live and dead spermatozoa with detached acrosomes decreased significantly ($P<0.01$) with the successive time of incubation. Similar trends were reported by Zeidan *et al*. (1998).

**Conception rate**

Data presented in Table 5 show that, the conception rates were 57.10 and 54.09 % for cows artificially inseminated with the frozen-thawed young and old bulls semen, respectively, without significant differences. These results reveal that the fertilizing efficiency of young bulls was insignificantly better than old bulls spermatozoa.

In conclusion, thawed-young bulls (3-5 years) semen supplemented with 10 mM caffeine showed better post-thawing motility, freezability, acrosome status and conception rate than old bulls (6-10 years) or free-caffeine medium. Therefore, it can be recommended to added 10 mM caffeine to the thawed-young or old bulls semen to improving the freezability and fertilizing ability when used for artificial insemination programme.

3

4

**Table 5: Effect of age on fertilizing ability of frozen bull semen.**

| Age | No. of cows inseminated | No. of cows conceived | Conception rate (%) |
|---|---|---|---|
| Young | 352 | 201 | 57.10 |
| Old | 342 | 185 | 54.09 |

**$X^2$ = 0.631 (not significant).**

## REFERENCES

Almquist, J.O. and R.P. Amann (1976). Reproductive capacity of dairy bulls. XI. Tuberal characteristics and post-puberal changes in production of semen and sexual activity of Holstein bulls ejaculated frequently. J. Dairy Sci., 59(5): 986-991.

Colas, G.(1975). The effect of initial freezing temperatures and addition of glycerol and dilution on the survival and fertilizing ability of deep frozen ram semen. J. Reprod. and Ferti., 42: 277-285.

Didion, B.A.; J.R. Dobrinsky; J.R. Giles and C.N. Graves (1989). Staining procedure to detect viability and the true acrosome reaction in spermatozoa of various species. Gamete Research, 22: 51-57.

Duncan, D.B. (1955). Multiple range and multiple F test. Biometrics, 11: 1-42.

El-Azab, E.A.; F.M. Labib; A.A. Selmi; H.E. Abbas (1998). Spermatozoal activities following caffeine or catalase addition to skim milk extended buffalo semen. Proc. 1 st Intern. Conf. Anim. Produ. & Health in Semi-Arid Areas, El-Arish, North-Sinai, Egypt, PP 177-186.

El-Gaafary, M.N. (1990). A diluent for frrzing of ram semen. Indian Journal of Animal Science, 60: 769-772.

Everett, R.W. and B. Bean (1982). Environmental influences on semen output. J. Dairy Sci., 65(7): 1303-1310.

Garbers D.L. and G.S. Kopf (1980). The regulation of spermatozoa by calcium and cyclic nucleotides. In: Advances in Cyclic Nucleotide Research, 13, Greengard, P. and Robinson, G.A. (Eds), New York, Raven Press, PP 251-306.

Garbers, D.L.; W.D. Lust; N.L. First and H.A. Lardy (1971). Phosphodiestrase inhibitors and cyclic nucleotides on sperm respiration and motility. Biochemistry, 10: 1825-1831.

Humphrey, J.D. and P.W. Ladds (1975). Chandes in the bovine testis and epididymis associated with age: A quantitative histological study. Res. Vet. Sci., 19: 135-141.

Jones, R.C. and D.L. Stewart (1979). The effects of cooling to 5ºC and freezing and thawing on the ultrastructure of bull spermatozoa. J. Reprod. and Fert., 56: 233-238.

Lenz, R.W.; C.N. Graves and J.R. Lodge (1977). Influence of incubation in seminal plasma on subsequent metabolic and morphological characteristics of bovine spermatozoa. Theriogenology, 7: 265-275.

Lindemann, C.B. (1978). A c,AMP-induced increase in the motility of dimembranated bull sperm models. Cell, 13: 9-18.

Maule, J.P. (1962). The Semen of Animals and Artificial Insemination. Tech. Comm., 15 Commonwealth Agricultural, Franhman Royal, England, UK, PP 420-430.

Miyamoto, H. and Y. Nishikawa (1979). Effect of caffeine on motility of bull spermatozoa. Japanese J. Zootechemical Sci., 50: 601-608.

Patt, J.A. and J. Nath (1969). Effects of diluents, equilibration time and freezing rates on the storage of ram semen. Criobiology, 5: 385-392.

Schoenfeld, C.; R.D. Amelar and L. Dubin (1975). Stimulation of ejaculated human spermatozoa by caffeine. Ferti. And Steril., 26: 158-161.

Simpson, A.M. and I.G. White (1987). Interrelationshipes between motility, cAMP, respiration and calcium uptake of ram and boar sperm. Anim. Reprod. Sci., 15: 189-207.

Snedecor, G.W. and W.G. Cochran (1982). Statistical Methods. 5 th Eddition, Iowa State University Press, Ames, USA.

Tash, J.S. and A.R. Means (1983). Cyclic adinosine 3'5' monophosphate, calcium and protein phosphorylation in flagellar motility. Biol. Reprod., 28: 75-104.

Tingari, M.D.; A.S. Ramos; E.S. Gaili; B.A. Rahma and Soad, A.H. (1993). Morphology of the testis of the one-humped camel in relation to age and reproductive activity. J. Anatomical, 139: 133-143.

Toconiz, J.F.; J. Betran; H. Bastidas; H. Larreal and P. Bastidas (1991). Testicular development, body weight changes, puberty and semen traits of growing Guzerat and Nellore bulls. Theriogenology, 35 (4): 815-826.

Zeidan, A.E.B.(1994). New aspects in freezing cattle semen. Ph. D. Thesis, Faculty of Agriculture, Zagazig University, Zagazig, Egypt.

Zeidan A.E.B.; M.N. El-Gaafary and F.E. El-Keraby (1998). Effects of new packaging method for frozen-bull semen in pellets form on some biochemical changes and conception rate. Proc. 1 st Intern. Conf. Anim. Produ. & Health in Semi-Arid Areas, El-Arish, North-Sinai, Egypt, PP 223-234.

# تحمل التجميد، حالة الكروموسوم ونسبة الخصوبة للحيوانات المنوية للطلائق عند الأعمار المختلفة مع اضافة الكافيين

**عبدالستار عبدالعزيز شتا**
**معهد بحوث الإنتاج الحيوانى – الدقى – الجيزة – مصر**

أجريت الدراسة على عدد 5 طلائق فريزيان صغيرة السن (3-5 سنوات)، 5 طلائق كبيرة السن (6-10 سنوات). تم جمع وتجفيف السائل المنوى لهذه الطلائق بمخفف الترس-فركتوز وكان معدل التخفيف 1 سائل منوى: 20 مخفف مع التبريد إلى درجة ºم5 فى غضون ساعتين ثم التجميد فى القصيبات البلاستيك مع الإسالة والتحضين على درجة ºم37 لمدة 4 ساعات مع اضافة الكافيين بمعدل صفر، 10 مللى مول/100 مل. تم تقدير حيوية الحيوانات المنوية وكذا قدرتها على تحمل التجميد وحالة الأكروسوم. كذلك تم قياس نسبة الخصوبة للسائل المنوى المجمد بعد الإسالة للطلائق الصغيرة والكبيرة السن.

وقد أظهرت النتائج أن السائل المنوى المجمد للطلائق الصغيرة السن كان أفضل بدرجة معنوية (على مستوى 1%) فى زيادة النسبة المئوية لحيوية الحيوانات المنوية وكذا قدرتها على تحمل التجميد من الطلائق الكبيرة السن. كذلك فإن إضافة 10 مللى مول كافيين إلى السائل المنوى المجمد بعد الإسالة والتحضين على درجة حرارة ºم37 لمدة 4 ساعات كان لها تأثيرا معنويا (على مستوى 1%) فى زيادة النسبة المئوية لحيوية الحيوانات المنوية وكذا قدرتها على تحمل التجميد وذلك فى الطلائق الصغيرة أو كبيرة السن. كان لفترة التحضين تأثيرا معنويا (على مستوى 1%) على انخفاض النسبة المئوية للحيوانات المنوية وكذا قدرتها على تحمل التجميد سواء فى الطلائق الصغيرة أو كبيرة السن. انخفضت النسبة المئوية للحيوانات المنوية الحية والمتماسكة الأكروسوم معنويا (على مستوى 1%) بينما زادت النسبة المئوية للحيوانات المنوية الميتة والمتماسكة الأكروسوم معنويا (على مستوى 1%) فى حين انخفضت نسبة الحيوانات المنوية الحية والميتة والمنزوعة الأكروسوم معنويا (على مستوى 1%) مع زيادة فترة التحضين. زيادة النسبة المئوية للحيوانات المنوية الحية سواء المتماسكة أو المنزوعة الأكروسوم فى الطلائق الصغيرة بدرجة غير معنوية عن الطلائق الكبيرة السن فى حين انخفضت النسبة المئوية للحيوانات المنوية الميتة سواء المتماسكة أو المنزوعة الأكروسوم معنويا (على مستوى 1%) عن الطلائق الكبيرة السن. اضافة 10 مل مول كافيين إلى السائل المنوى بعد الإسالة أدى إلى زيادة نسبة الحيوانات المنوية الحية سواء المنزوعة أو المتماسكة الأكروسوم بدرجة غير معنوية مع انخفاض النسبة المئوية للحيوانات المنوية الميتة سواء المنزوعة أو المتماسكة الأكروسوم معنويا (على مستوى 1%) مقارنة بالسائل المنوى بدون إضافة الكافيين.

زيادة معدل الخصوبة للأبقار الملقحة صناعيا بالسائل المنوى المجمد للطلائق الصغيرة بدرجة غير معنوية عن الطلائق الكبيرة السن.

**Table 1: Effect of bull age, caffeine supplementation and incubation time on post-thaw sperm motility of Friesian bulls.**

| Incubation time (hr) | Age | | | | | |
|---|---|---|---|---|---|---|
| | Young | | Overall means | Old | | Overall means |
| | Control | Caffeine | | Control | Caffeine | |
| 0 | 41.32±3.27 | 48.28±3.30 | 44.80±3.48$^{a}$ | 37.50±2.15 | 41.72±1.18 | 39.61±2.11$^{a}$ |
| 1 | 37.48±2.30 | 65.20±2.76 | 51.34±3.86$^{b}$ | 30.15±2.20 | 58.62±1.28 | 44.39±14.24$^{b}$ |
| 2 | 32.15±2.03 | 58.46±3.08 | 45.31±13.16$^{c}$ | 19.25±1.18 | 49.80±2.35 | 34.53±15.28$^{c}$ |
| 4 | 18.74±3.25 | 40.62±2.75 | 29.68±10.94$^{d}$ | 14.32±2.15 | 32.92±1.72 | 23.62±9.30$^{d}$ |
| Overall means | 32.42±4.93$^{b}$ | 53.14±5.43$^{a}$ | 42.78$^{A}$ | 25.31±5.24$^{b}$ | 45.77±5.50$^{a}$ | 35.54$^{B}$ |

**Means bearing different letters within the same classification, differ significantly (P<0.01).**

**Table 2: Effect of age, caffeine supplementation and incubation time on freezability of Friesian bull semen.**

| Incubation time (hr) | Age | | | | | |
|---|---|---|---|---|---|---|
| | Young | | Overall means | Old | | Overall means |
| | Control | Caffeine | | Control | Caffeine | |
| 0 | 51.65±3.60 | 60.35±2.55 | 56.00±4.35$^{a}$ | 46.88±2.61 | 52.15±3.50 | 49.52±2.64$^{a}$ |
| 1 | 46.85±2.84 | 81.50±2.46 | 64.18±17.33$^{b}$ | 37.69±2.85 | 73.28±3.26 | 55.49±17.80$^{b}$ |
| 2 | 40.19±2.65 | 73.08±2.72 | 56.64±16.44$^{c}$ | 24.06±2.42 | 62.25±2.45 | 43.16±19.10$^{c}$ |
| 4 | 23.43±3.16 | 50.78±2.84 | 37.11±13.68$^{d}$ | 17.90±2.90 | 41.15±2.84 | 29.53±11.63$^{d}$ |
| Overall means | 40.53±6.17$^{b}$ | 66.43±6.79$^{a}$ | 53.48$^{A}$ | 31.63±6.55$^{b}$ | 57.21±6.88$^{a}$ | 44.42$^{B}$ |

**Means bearing different letters within the same classification, differ significantly (P<0.01).**

**Table 3: Effect of age, caffeine supplementation and incubation time percentage of live intact (HA) and detached (LDA) acrosomes of bull spermatozoa.**

| Incub. times (hr) | Age | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Young | | | | | | Old | | | | | |
| | Control | | Caffeine | | Overall means | | Control | | Caffeine | | Overall means | |
| | LIA | LDA | LIA | LDA | LIA | LDA | LIA | LDA | LIA | LDA | LIA | LDA |
| 0 | 30.82±2.34 | 4.65±1.78 | 32.65±2.30 | 4.82±2.75 | 31.74±0.92a | 4.74±0.09b | 27.80±2.12 | 3.72±2.31 | 30.11±2.30 | 4.20±2.33 | 28.96±1.16a | 3.96±0.24c |
| 1 | 29.35±2.42 | 4.92±2.54 | 30.72±2.43 | 5.14±2.66 | 30.04±0.69a | 5.03±0.11b | 24.65±2.50 | 3.9±2.75 | 28.4±2.65 | 4.28±2.40 | 26.53±1.88a | 4.09±0.19b |
| 2 | 23.76±2.60 | 5.22±2.36 | 24.36±2.80 | 5.90±1.88 | 24.06±0.30b | 5.56±0.34a | 20.74±2.72 | 4.78±2.46 | 23.86±1.92 | 4.65±2.64 | 22.3±1.56b | 4.72±0.07b |
| 4 | 14.60±2.16 | 5.90±2.48 | 17.82±2.75 | 5.86±2.34 | 16.21±1.61c | 5.88±0.02a | 14.56±1.04 | 5.68±1.87 | 16.32±2.84 | 5.24±2.53 | 15.44±0.88c | 5.46±0.22 |
| Overall means | 24.63±3.67 | 5.17±0.27 | 26.39±3.36 | 5.43±0.27 | 25.51 | 5.30 | 21.94±2.85 | 4.52±0.45 | 24.67±3.08 | 4.59±0.24 | 23.31 | 4.56 |

**Means bearing different letters within the same classification, differ significantly (p<0.01).**

**Table 4: Effect of age, caffeine supplementation and incubation time percentage of dead intact (DIA) and detached (DDA) acrosomes of bull spermatozoa.**

| Incub. Time (hr) | Age | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Young | | | | | | Old | | | | | |
| | Control | | Caffeine | | Overall means | | Control | | Caffeine | | Overall means | |
| | DIA | DDA | DIA | DDA | DIA | DDA | DIA | DDA | DIA | DDA | DIA | DDA |
| 0 | 39.7±2.25 | 25.18±2.98 | 36.12±2.17 | 26.78±2.64 | 37.91±1.79c | 25.98±0.80c | 41.22±1.92 | 26.16±2.75 | 40.28±2.16 | 28.15±3.04 | 40.75±0.47c | 27.16±1.0c |
| 1 | 40.51±2.34 | 26.34±3.01 | 38.17±2.34 | 28.46±2.18 | 39.34±1.17c | 27.50±0.96c | 43.24±2.46 | 28.62±2.6 | 41.12±2.45 | 29.17±2.18 | 42.18±1.06c | 28.9±0.28c |
| 2 | 44.15±2.65 | 27.16±2.18 | 41.28±2.4 | 30.85±2.15 | 42.72±1.44b | 29.01±1.85b | 47.18±2.65 | 31.18±2.19 | 42.54±2.33 | 34.28±2.46 | 44.86±2.32b | 32.73±1.55b |
| 4 | 48.6±3.2 | 31.14±2.42 | 47.25±2.65 | 34.56±2.84 | 47.93±0.68a | 32.85±1.71a | 51.26±3.10 | 36.24±3.02 | 46.42±2.18 | 39.2±2.4 | 48.84±2.42a | 37.72±1.48a |
| Overall means | 43.24±2.03A | 27.46±1.29b | 40.71±2.43B | 30.16±1.69a | 41.98B | 28.83B | 45.73±2.22A | 30.55±2.16b | 42.59±1.36B | 32.7±2.55a | 44.16A | 31.63A |

**Means bearing different letters within the same classification, differ significantly (p<0.01).**