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Induction of Triploidy in Indian Edible Oyster *Crassostrea madrasensis* (Preston) Using 6-Dimethylaminopurine

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Abstract

Triploidy was induced in the Indian edible oyster *Crassostrea madrasensis*, by treating the newly fertilized eggs with 6-Dimethylaminopurine.The effect of different concentrations and durations of treatment on triploidy induction and survival of embryos were examined. The optimum condition was found to be 100 µM concentration of 6-DMAP for 8 minutes when 50% of the freshly fertilized eggs have extruded the first polar bodies. This yielded 67±1.7 % of triploid on the first day and 62±1.5% on the D-stage larvae as determined by examination of the metaphase chromosomes. Survivability among the different treatment groups at D stage were not significantly different. This is the first demonstration of induction of triploidy in *Crassostrea madrasensis* using 6-DMAP and since edible oyster farming is becoming increasingly popular in India, application of this technique in the production of culture stock assumes importance.

Introduction

Induction of triploidy has been proposed as a means for enhancing growth rate and meat quality in bivalve mollusks. Stanley et al. (1981) successfully induced triploidy for the first time in oyster using cytochalasin-B (CB).This was followed by other reports of induction of triploidy in oysters and clams.(Allen et al. 1989; Dufy and Diter 1990).More recently, Nell (2002) reported successful induction of triploidy in *Crassostrea gigas*, *Crassostrea virginica*, *Saccostrea glomerata* and *Ostrea edulis*. Although *Crassostrea madrasensis* is the dominant Indian edible oyster species under cultivation, there has been no attempt to induce triploidy in them.

The method generally employed for the induction of triploidy is by blocking the extrusion of the second polar body through the interference with the second meiotic division of the freshly fertilized eggs. Various agents have been used to induce triploidy in bivalve molluscs. Physical agents used are heat, cold and pressure shock while the chemical agent extensively used is CB. Scarpa et al. (1994) compared six triploidy inducers (CB, heat, calcium, caffeine, combined calcium and heat and combined caffeine & heat), and reported that CB was the most effective. Of late, the purine analogue viz 6-Dimethylaminopurine (6-DMAP) has been

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proposed as a better choice for induction of triploidy. Initially it was used as a cleavage inhibitor in sea urchin eggs (Rebun et al. 1973). Subsequent studies by Neant and Guerrier (1988) demonstrated that this is an inhibitor of protein phosphorylation, which seems to act on specific kinases and promote chromatin decondensation. Simultaneously, it also exerts its action on microtubule organization (Desrosiers et al. 1993). It is known to block the extrusion of polar body in a number of marine invertebrates as well as mouse oocytes (Szollosi et al. 1991) hence it can be used to induce triploidy. Suppression of second polar body formation for induction of ploidy in bivalves using 6-DMAP has been achieved by (Gerard et al. 1994; Nell et al. 1996; Zhang et al. 1998; Norris and Preston 2003). Use of 6-DMAP is simple, as it does not require expensive or specialized equipment for application. It is water-soluble and can be easily washed off from the embryo after treatment. It is easy to handle, safer than CB and is not a carcinogen.

This paper describes the results of the experimental induction of triploidy in *C. madrasensis* using 6-DMAP at 29ºC, the typical ambient temperature in this tropical region.

Materials and Methods

Gamete collection and fertilization

Mature *C. madrasensis* were collected from both natural beds and the oyster farm of Central Marine Fisheries Research Institute at Tuticorin. Samples of 10 oysters were opened to determine the gonadal maturity. Spawning in ripe oyster was induced by thermal stimulation at 32^0C. At the time of spawning, female and male oysters were taken out and kept separately in 500 ml spawning trays with filtered seawater. On completion of spawning, oysters were removed from the beaker. Four females and two males were used in each experiment. The eggs were pooled and filtered through 100µm sieve to remove debris.

The eggs were fertilized by adding the sperm. Diluted suspension of sperms was used as this would facilitate their dispersion and promote synchronous development .Kinetics of polar body extrusion was monitored by viewing the freshly fertilized eggs under light microscope. Time needed for the release of 50% of the first polar bodies was found to be 16 minutes after fertilization, at room temperature (29ºC). Nell et al. (1996) reported that the ideal time of initiation of treatment is when 50% of polar bodies have been extruded.

Triploidy induction

Freshly fertilized eggs were exposed to four concentrations of 6-DMAP and three durations of exposure to determine the optimum dose and duration of treatment. The treatment was initiated during the 17^{th} minute post fertilization. The fertilized eggs were incubated with 6-DMAP at different concentrations, viz., 0 (Control), 50, 100, 150 and 200 µM (0, 8.15, 16.3, 24.5, 32.6 mg•l^{-1}). Three durations of exposure viz., 5, 8 and 10 minutes were tried for each dose. The treatment was carried out at ambient seawater temperature (29ºC).

For this fertilized eggs were collected using 20 µm filter. The eggs thus collected were divided into 13 aliquots of which one was used as control while each of the remaining were subjected to a particular treatment. Treatment was effected by dipping the filter with the eggs into appropriate concentrations of 6-DMAP for the required duration.

Larval rearing

Following the treatment, the larvae were reared in filtered seawater (32 ppt salinity) at a density of about 50 larvae•ml^{-1} in plastic tubs at room temperature. The effect of 6-DMAP was monitored in treated larvae. The larvae were fed with a diet of Isochrysis galbana until the pedivelliger stage, and thereafter on a mixed culture of *I. galbana* and *Chaetoceros calcitrans* till the spat settled. Algal culture was filtered through a 92 µm sieve till the larvae grew to spat and then through a 120 µm sieve. Every alternate day the seawater was fully changed. The larval density was noted on the first, second and fifth day and larval survival was estimated for the treated groups as well as control. Egg number prior to fertilization was also noted using a Sedgewick Rafter slide.

Ploidy determination

Percentage of triploid larvae on the first day and D stage larvae on the fourth day were determined by chromosome counts in metaphase plates prepared from them following Allen et al. (1989) with modifications. The modified technique for the first day larvae consisted of exposure of 5 ml of larval sample to 0.02% colchicine for 1hr followed by hypotonic treatment in 10 ml of 50% seawater. Seawater was pipetted out after 20 min followed by treatment with Carnoy's fixative (3:1, absolute methanol and glacial acetic acid (9.5% assay) respectively) for 5 minutes. Treatment with Carnoy's fixative was repeated 3 more times at 10, 15 and 20-minute intervals each time. Afterwards, a few drops of 50% glacial acetic acid (refrigerated) was added to the embryos and thoroughly mixed. A drop of the suspension was dropped on a warm clean glass slide (50^{o}C). The slides were air dried, stained using giemsa in phosphate buffer (pH 6.8) for 20 minutes. For the preparation of metaphase plate from D stage larvae, about 50 larvae were treated with 0.02% colchicine for 1 hr. Approximately 5 ml of larvae were aspirated into a pipette and placed into several drops of seawater on a standard microscope slide. A second microscope slide was gently placed over the first, sandwiching the larvae between the two slides and crushed. The pair of slides with the larvae was then placed upright on its end and the larvae were allowed to fall onto the cavity block with the help of seawater. Larvae were examined under a dissecting microscope to check the degree of breakage and if insufficient, the procedure was repeated. After this, hypotonic solution was added into the cavity block and procedure for the fixation was continued as described earlier for the first day larvae. Chromosomes were counted under a microscope (40x).

The data were analyzed by ANOVA using SYSTAT software (version 7.0.1) and the ploidy percentage resulting from different concentrations and durations of 6-DMAP treatments were compared for the significance of difference.

Results

In the present study the initial trials using 300 µM of 6-DMAP with 5 and 10-min exposure time at 29ºC was found to be detrimental to embryos as only a few larvae survived after treatment. The treated larvae were seen in spiral motion leading to complete mortality by the second day. Therefore, lower concentrations (50, 100, 150 & 200 µM) were tried in subsequent trials and this resulted in the production of surviving triploid larvae. The percentage of triploidy induced by different concentrations and durations of exposure to 6- DMAP are given in Table 1. Among the different concentrations tried, 50, 100 and 150 µM applied for 8 min resulted in high triploid yield exceeding 60% on first day larvae while 200 µM yielded

46% only. The concentration of 100 µM applied for 8 min was found to be yielding, the highest percentage in both first day (66.6%) and 'D' stage larvae (61.8%).

Table 1. Relative efficiency of different concentration and durations of 6-DMAP exposures for induction of triploidy in the edible oyster *C.madrasensis**

Duration / Concentration	Percentage of triploid					
	Larval stage			D stage		
	5min	8min	10min	5min	8min	10min
50µM	35.26±1.50	60.03±2.01	55.00±1.63	33.43±1.42	46.07±2.74	53.06±1.84
100µM	45.65±1.36	66.60±1.65	46.20±2.11	42.20±1.56	61.82±1.48	40.55±1.96
150µM	57.14±1.60	61.82±2.09	44.52±1.50	55.55±1.75	55.00±1.40	40.18±2.01
200µM	58.75±2.57	46.34±1.50	42.15±2.31	47.05±2.57	41.70±1.44	40.75±1.58

Note: The concentration, duration and interaction of treatment and duration differ significantly ($p<0.01$)
*Values are means ± SE (n=3)

Survivability of larvae exposed to various concentrations of 6-DMAP for 8 minutes are presented in Table 2. On the first day, maximum survival was observed with 50 µM (61%), the survival rate decreasing with a increase in the concentration. However, survival in 100 µM treatment group, which was adjudged as optimum for induction of triploidy, was lower by only 1.48%. By the fifth day the difference in survival rate of various treatment groups narrowed down to an insignificant level.

Table 2. Percentage survival of *C. madrasensis* larvae exposed to different concentration of 6-DMAP for 8'

Treatment	Days		
	First	Second	Fifth
Control	71.00	62.14	44.28
50 µM	61.00	39.52	20.00
100 µM	59.52	39.28	19.04
150 µM	49.28	21.42	18.57
200 µM	46.42	23.57	17.14

Discussion

This work is the first report on production of triploid *C. madrasensis* using 6-DMAP and the first demonstration of a triploid shellfish in India .The procedure for producing triploidy in *C. madrasensis* using 6-DMAP was found to be simple as has been reported by Gerard et al. (1994) in Pacific oysters. Although concentrations of 300 µM and 450 µM were recommended for Pacific oysters by Desrosiers et al. (1993) and Gerard et al. (1994), and concentrations of 200 µM to 400 µM were reported to be ideal in *S. commercialis* by Nell et al. (1996), we observed that doses of 300 µM were detrimental in *C. madrasensis* probably due to the effect of a higher treatment temperature. Zhang et al. (1998) reported that in *Haliotics discus hannai*, no veliger larvae could survive when 6-DMAP was used at 300 µM and beyond. Liu et al. (2004) also reported that 6-DMAP concentrations of 200 and 250 µM led to unacceptable larval survival of *Haliotis rubra.* According to them 6-DMAP at higher concentrations disrupts metaphase spindles too quickly with the result that only fewer eggs are able to complete metaphase-anaphase transition during treatment than at lower concentrations.

Percentage of triploid larvae realized in this study from exposure to 200 µM is comparable with that of *Saccostrea commercialis* reported by Nell et al. (1996). The percentage of triploids on first day larvae and five day old larvae reported by them were 53 and 49 as against 59 and 47 on the first day and fourth day larvae realized in this study.

Among the four concentrations, each applied for three durations, the dosage of 100 µM applied for 8 min was found to be optimum, with the highest percentage of triploid larvae,

as against 300 µM and 450 µM recommended for Pacific oysters by Desrosiers et al. (1993) and Gerard et al. (1994). However, this is in agreement with the finding of Liu et al. (2004) that 100 µM is the optimal concentration of 6-DMAP for induction of triploidy in *H. rubra*. The trend was the same in repeat trials. This treatment yielded 67% triploidy on the first day. Percentage of triploids in the D stage larvae on the fourth day (62%) was lower than on the first day larvae, probably due to the enhanced mortality resulting from abnormal development in some larvae consequent to aneuploidy as suggested by Nell et al. (1996) for *S. commercialis*.

Although survival rate decreased with increasing concentrations on the first day, the differences narrowed down by the fifth day. Although, maximum survival rate was observed with 50 µM, it was not significantly different from that of 100 µM treatment, which was adjudged as optimum for induction of triploidy.

Conclusion

The 6-DMAP has been reported to be less hazardous than CB which is extensively used at present for induction of triploids (Desrosiers et al. 1993). The 6-DMAP treatment involves only a single brief exposure of the fertilized eggs. As the treatment involves exposure of the zygote only, possibility of chemical residue in the adult bivalve used for human consumption does not arise (Nell et al. 1996). Hence 6-DMAP can be safely recommended for the production of triploid *C. madrasensis*. Since 100 µM can induce surviving triploids even at 29ºC, this can be used by those who do not have a sophisticated laboratory facility.

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