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NOTE

Triploidy induction and confirmation in the edible oyster *Crassostrea madrasensis*

P.C.Thomas, Jyothi V.Mallia and P.Muthiah

Central Marine Fisheries Research Institute, Cochin 682 018, India

Abstract

This paper presents the protocols standardized for producing triploid edible oyster, *Crassostrea madrasensis* by retention of II polar body (MII triploids), using physical (heat and cold shocks) and chemical agents (Cytochalasin B and 6-Dimethylaminopurine), as well as the protocol for preparation of metaphase spreads for cytological confirmation of triploidy and estimation of its percentage. Among the four inducing agents tried, 6-Dimethylaminopurine yielded highest percentage of triploids (66.6 in day old and 61.82 in "D" stage larvae).

Ploidy manipulation in bivalves has received much attention, mainly because of its potential advantages in aquaculture (Beaumont and Fairbrother, 1991). Induction of triploidy has been proposed as a means for enhancing growth rate and meat quality. Triploids can be produced by applying a physical or chemical shock to the newly fertilized eggs during meiosis. This leads to the retention of the I or II polar body resulting in the production of Meiosis I (M I) or Meiosis II (M II) triploids respectively. Stanley *et al.* (1981) first attempted triploidy induction in oyster (*Crassostrea virginica)* successfully. Thereafter, triploidy has been induced in *C. gigas, C.virginica, Saccostrea glomerata* and *Ostrea edulis* (Nell 2002). In India, commercially important edible oyster *C.madrasensis* is distributed all along the east and west coasts. By establishing full-fledged shellfish hatchery, at Tuticorin, the Central Marine Fisheries Research Institute (CMFRI) has initiated the mass production of edible oyster seed through hatchery systems (Nayar *et al.*, 1987). The edible oyster farming technology developed by the Institute has been successfully adopted by fishermen (Appukuttan, 2001). The farming is becoming increasingly popular in India. Therefore, application of techniques like ploidy manipulation for the production of superior culture stock assumes importance. This paper presents the protocols optimized for induction of triploidy in the Indian backwater oyster, *C.madrasensis* with various inducing agents for the first time.

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Material and methods

Collection and conditioning of oysters

Mature oysters of size range 60-90 mm collected from natural beds and oyster farms were used for the present study. Samples of 10 oysters were opened for checking the gonad maturity stage and the mature ones were induced for spawning. Those in the maturing stage were kept in the conditioning room, where they were intensively fed with mixed algal culture at 22 ± 1°C (Nayar *et al.*, 1987). After 10-15 days on assessing the gonadal condition the oysters were used for induced spawning experiments.

Brood stock conditioning plays an essential role in successful production of triploids. Ripe eggs uniformly conditioned for fertilization will go through meiosis synchronously and arrive together at the time of treatment. Unripe eggs will produce asynchronous finish. Synchrony is important in the eggs collected from different females. Brood stocks of fully mature males were selected so that the sperms were not only active but will fertilize the egg instantaneously to promote synchronous development. All the females should be at the same stage of maturity to ensure synchrony of development in the eggs on pooling.

Induction of spawning

Gametes were collected by natural spawning or by stripping the mature oysters. For this animals were maintained in the wet laboratory and trials were conducted to standardize protocols for induced spawning through heat shock, manipulation of *p*H as well as through chemical treatment with Tris buffer. The mature oysters were thoroughly washed and transferred to a 100-litre perspex-spawning tank containing about 50 litres of seawater (31ppt) maintained at a temperature of 2-4°C above the ambient condition with proper aeration. If spawning did not occur within an hour, fresh sperms stripped from a sexually ripe male were introduced in the tank containing the brood stock to induce sympathetic spawning. When an oyster started spawning it was transferred to a glass tray containing filtered seawater. Only one oyster was placed in each tray. Individual oysters were allowed to complete the spawning in the tray. The gametes in the trays were filtered through a 100μm sieve into 10 litre glass beakers. At this stage mild aeration was given to ensure sufficient supply of oxygen.

Care was taken to prevent inadvertent contamination (fertilisation). Egg density was noted before fertilization. Eggs were counted and suspended in half the correct final volume of seawater. For counting, 1ml of liquid from the egg container was taken (whose volume is about 5 litres) and diluted with 1 litre of seawater. From this, 1ml was placed in a Sedge Wick Rafter Slide and the eggs were counted under a microscope. The procedure was repeated to confirm the accuracy of the first count.

In vitro fertilization

The eggs were fertilized by adding the sperms. Diluted suspension of sperms was

used as this would facilitate their dispersion and promote synchronous development.

On perfecting the induced spawning and *in vitro* fertilization, the experimental trails were carried out to study

1. kinetics of 1st and 2nd polar body extrusion and time of first cleavage to determine the appropriate time for initiation of triploidy induction treatment and
2. protocols for preparing metaphase spreads from first day larvae and D-stage larvae.

Triploidy induction trials

The method generally suggested 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 indicated for the induction of triploidy in bivalve molluscs. Physical agents used were heat, cold and pressure shock while the chemical agent were Cytochalasin B (CB) and 6-Dimethylaminopurine (DMAP).

In the present study freshly fertilized eggs were exposed to all the above agents to arrest II PB extrusion and induce triploids. Trials were carried out with different concentrations of DMAP and CB and different temperatures with varying duration of exposure to optimize the protocol for inducing triploidy with each of them. Treatment was effected by collecting the fertilized eggs in 20μm mesh filter and the dipping the same in appropriate concentrations of the inducing agent for the required duration. Triploids were identified by the larval chromosome count in the metaphase plates.

Results and discussion

Protocol for induction of triploidy

Oysters in the size range 60-90 mm of which about thirty percent belonging to zero year class or just one year old are recommended for the programme. The ideal time suggested for treatment of the freshly fertilized egg for arresting second polar body and inducing triploidy is when 50% of first polar bodies have been extruded. In the present study the extrusion of 50% first polar body was achieved 16minutes post fertilization at 28-29ºC (normal room temperature). Hence treatments were initiated 17 minutes post fertilization to arrest II PB extrusion using both physical and chemical agents. General scheme of the protocol optimized in the present study for induction of triploidy in edible oyster embryo included the following steps:

1) collection/conditioning of gravid oyster,
2) collection of gametes,
3) sieving through 100μ,
4) artificial fertilization,
5) rinse with seawater and sieve through 20μ,
6) dipping the sieve containing fertilized egg in 6-DMAP (100μ M)8'/CB (0.05mg/l)3'/Heat (37°C) 5'/Cold (5°C) 10', and
7) washing with filtered seawater.

The optimum dosage and duration of treatment standardized for each of the inducing agents are presented in Table 1.

Protocol for metaphase spread and ploidy determination

The technique for preparation of metaphase plate consisted of exposure of 5 ml of the larval sample to 0.02% colchicines for 1 hr in a cavity block, followed by their transfer to 10 ml of 50% seawater for hypotonic treatment. After 20 minutes the seawater was pipetted out, few drops of Carnoy's fixative was added. After five minutes the supernatant fluid was removed. The process was repeated for three more times at 10,15 and 20 minute intervals each time. Finally a few drops of 50% cold acetic acid was added and mixed thoroughly by aspirating and ejecting using a Pasteur pipette. Finally a drop of suspension was dropped on a warm cleaned glass slide (50°C) placed on a slide warmer using Pasteur pipette. The slides were air dried and stained in Giemsa in phosphate buffer (*p*H 6.8) for 20 minutes. Chromosomes were counted under microscope (40x magnification). The triploid metaphase number is 30 (Fig.1). The following ploidy classification is applied <18 neglected, 18-21 diploid, 25-34 triploid, 35-44 tetraploid. (Yamamoto *et al.*, 1988). A wide range was used to account for artificial loss of chromosomes and overlapping cells, and to give a more realistic estimate of ploidy rather than omitting such cells from analysis (Guo *et al.*, 1992; Shen *et al.*, 1993). For the preparation of metaphase plate from D stage,

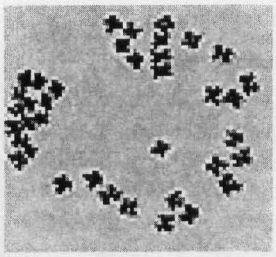

Fig.1. *Metaphase spread of triploid* in C.madrasensis

about 50 larvae were treated with 0.02% colchicine for 1 hr. Approximately 5 ml of larvae were drawn into a Pasteur 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,ccc hypotonic solution was added into the cavity block and procedure for the fixation was continued as described earlier.

Table 1. *Dosage and duration of treatment for optimum triploidy in* C.madrasensis

Inducing agent	Concentration / Temperature	Duration in minutes
Heat	37ºC	5
Cold	5ºC	10
CB	0.05mg/l	3
6-DMAP	100μm	8

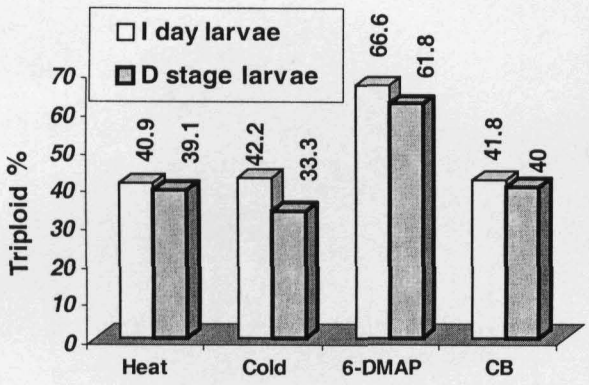

Fig. 2. *Relative efficiency of different treatments for induction of triploidy in* C.madrasensis

Ploidy inducing efficiency of various agents

Percentage of triploid larval yields estimated for different physical and chemical agents are presented in Figure 2. Among the various physical and chemical treatments tried for producing II meiotic triploids, 6-DMAP yielded the highest percentage of 66.6% at larval stage and 61.8% at 'D' stage. This result indicated that 6-DMAP is ideal for inducing triploidy in *C.madrasensis* at 29°C.

Larval rearing

Triploid larval culture is same as normal diploid culture. According to Allen (1986) it is more important for triploid larvae, that the water be changed at the straight hinge stage (by day 2) because more of the treated larvae are expected to die and therefore produce more extraneous organic matter. Following the treatment, the larvae are to be reared in filtered seawater (32ppt) at a density of about 50 larvae/ml in plastic tubs at room temperature. Till the pedivelliger stage, the larvae may be fed with a diet of *Isochrysis galbana* and thereafter a mixed culture of *Isochrysis* and *Chaetoceros.* Algal culture filtered through 92μ sieve may be fed till the larvae grow to spat and then through 120μ sieve. Every alternate day the seawater was fully changed.

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