

## ***In vitro* sterilization technique on embryo of black Toraja rice**

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**Abstract.** Toraja black rice has a high anthocyanin content, a water-soluble pigments, with antioxidant activity. Toraja black rice has a variety of seeds colour in one panicles such as full black (the outside and inside the rice), medium black (the outside and slightly inside rice) and a little black (only the outside of rice). Embryo culture *in vitro* is one way to grow plants in sterile conditions. The presence of contamination and the death of the embryo require *in vitro* embryo culture. The sterilization technique is a very important first step to eliminate contamination and the death of embryos. This research aims to determine the right material composition for sterilization of black rice's embryo. The experiment was done by growing black rice on half strength MS media with the treatment of three method of sterilization, *i.e.*: S1 (70% alcohol for 5 minutes, 3% and 2% Chlorox each for 10 minutes), S2 (70% alcohol for 3 minutes, 2% Clorox for 10 minutes) and S3 (70% alcohol for 3 minutes and 1% Clorox for 15 minutes). The materials used are rice seedlings that have been cut in two and opened the pericarp of paddy grain, leaving a piece of rice that has a complete embryo. The best sterilization for Toraja black rice embryo culture was using the S3 composition. Best germination was seen on the seeds with full and medium black color.

### **1. Introduction**

Black rice from Toraja District / North Toraja region, has a unique phenotype character. A dark purple color that makes this rice looks black at a glance, caused by high anthocyanin content. Anthocyanins are water-soluble pigments, which have antioxidant activity. Black rice is known to increase the body's resistance to disease, repair damaged liver cells, prevent renal function, prevent cancer / tumor, slow aging, as an antioxidant, clean cholesterol in the blood, and prevent anemia [1, 2].

Black rice in Indonesia is predominantly derived from local rice from various regions. One of the areas in Indonesia that has a variety of local rice is South Sulawesi. Local rice is a major source of genetic diversity in rice breeding efforts, especially black rice. One of the local rice varieties used for generations as part of the tradition, ritual and culture of the community is black rice (Pare Ambo) from Toraja District / North Toraja District.

The productivity of local paddy especially Toraja black rice is still low. BPS data [3] noted that local rice productivity in the country of 3.99 tons ha<sup>-1</sup> is much lower than that of paddy field productivity of 5.26 tons ha<sup>-1</sup>. Other shortcomings due to black Toraja rice have a deep age, ranging from 5-6 months [4]. This cause the black rice is less developed in a broad scale.

In addition, Toraja black rice has a very low level of purity, to be used as a source of genetic diversity. Preliminary studies have been done, indicating that at one panicle there were different colors of seeds.



Some black seeds are black in whole (the outside and in the rice), others are medium black (the outside and slightly inside the rice) and are black in color (only the outside of the rice). In vitro culture has been widely used for the development of plant food cultivation. In plant breeding, embryo rescue culture applications are expected to maintain their integrity and grow into new plants [5]. One of the most widely used in vitro culture techniques is embryo culture, because embryo culture is one of the earliest studies to obtain plants that are free from contamination of microorganisms.

Through embryo culture, early embryo development can be studied. In the field of plant breeding, embryo culture can speed up the hybridization cycle [6]. The choice of embryos as explants due to the availability of seeds, has a high physiological uniformity and can be carried over time and at considerable distance [7].

One obstacle in the process of embryo culture in vitro is contamination. The sterilization technique is a very important first step to eliminate contamination and the death of embryos in *in vitro* cultures. In addition it is expected that sterilization does not change the color of black rice rice.

## 2. Materials and methods

### 2.1. Place and Time

The research was conducted at the Laboratory of Plant Biosciences and Reproduction Biotechnology, Department of Agronomy, Faculty of Agriculture, Hasanuddin Universit, Makassar. The study took place from April to November 2017.

### 2.2. Materials

Black rice seed of Pare Ambo variety was obtained from farmers survey in Ao 'Gading Toraja Utara, inorganic and organic material components for the manufacture of Murashige and Skoog (MS) media, clorox and alcohol.

### 2.3. Implementation of Research

There were two goals in this experiment. First experiment was carried out by testing kinds of sterilization technique for Toraja's black seed and the second experiment was to see germination performance of black seeds of Toraja.

**2.3.1. Experiment 1.** Preparation of materials. The seeds used were peeled Toraja black rice seeds cut crosswise into two parts to see the color within the endosperm. The part used was the embryo half pieces. Peeling and cutting the seed were conducted to select and classify seeds based on the same color criteria. The pieces are grouped into three sections of the rice color level: the full black (HP) (black on the surface and inside of the rice), the medium black (HM) (black on the surface and slightly inside the rice) and the black one (HS) (black only the surface of the rice) (figure 1). The seeds of embryo half pieces are grown in vitro on  $\frac{1}{2}$  MS germination medium after a sterilization process.



**Figure 1.** Cross-section of black rice seeds of Toraja (a) Black rice seeds with black color (HS), (b) Black rice seeds with medium black color (HM), and (c) Black rice seeds with full black color HM).

Sterilization was performed with three sterilization sequence treatments based on the materials used. Treatment of S1 was done by sterilizing rice explants consecutively using 70% alcohol for 5 minutes, rinsed with sterile distilled water 3 times, then soak in 3% Chlorox solution for 10 minutes, rinsed again with sterile distilled water for 3 times, continued with immersion in 2% Chlorox for 5 minutes and finally rinsed with again with sterile distilled water 3 times. Sterilization S2 treatment with 70% alcohol for 1 minute, rinsed with sterile distilled water 3 times, followed by 2% Chlorox solution for 10 minutes, and finally rinsed with sterile aquades 3 times. S3 sterilization treatment using 70% alcohol for 3 minutes, then rinsed with sterile distilled water 3 times, followed by 1% clorox for 15 minutes, and finally rinsed with sterile distilled water 3 times. Quantitative and qualitative observations were made on the color of cut seeds with embryo part after sterilization process.

**2.3.2. Experiment 2.** Preparation of materials. The second experiment was based on the first experiment. Seed materials used were black rice seeds of Toraja black rice which have the embryo part and are full black rice (surface and inside of part rice), medium black (surface and slightly inside of part rice) and thin black (only the surface of rice). Half-cut seeds were further planted in vitro on germination medium  $\frac{1}{2}$  MS after going through the sterilization process. The sterilization process is based on the best results of the first experiment. Quantitative observations include observing the number of days required to begin germination (day), and the length of the plumula (cm).

#### 2.4. Methods and Analysis

The embryo culture in the laboratory consists of three treatment of sterilization sequence based on the materials used are S1, S2 and S3. Treatment S1 is a sequence of sterilization using alcohol 70% for 5 minutes, Chlorox 3% for 10 minutes and Chlorox 2% for 5 minutes. The treatment of S2 is sterilization with alcohol 70% for 1 minute and Chlorox 2% for 10 minutes. S3 treatment using alcohol 70% for 3 minutes and Chlorox 1% for 15 minutes. To know the difference of treatment, data was analysis by test of two middle value in pairs.

### 3. Result and discussion

#### 3.1. Result

**3.1.1. Sterilization experiment of black rice embryo.** Table 1 shows germination of Toraja black rice embryo in combination treatment of sterilization composition. The results of the experiment showed that each sterilization sequence technique can be used for sterilization of black Toraja rice with different germination rates. This can be seen from the absence of contaminants in the culture. The best sterilization composition for black rice embryo culture is S3 sterilization composition with 93% germination.

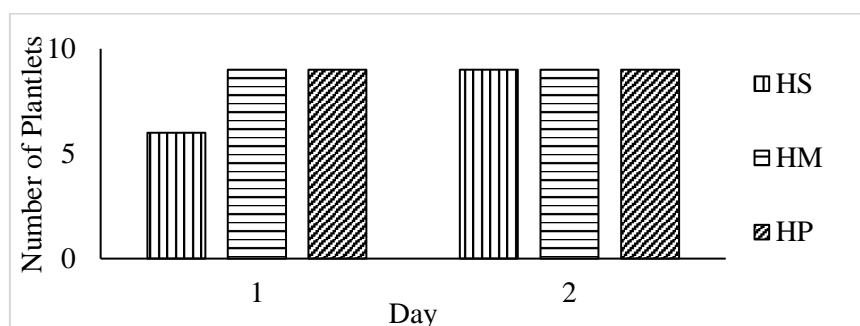
**Table 1.** Germination of black rice embryo of Toraja on treatment of combination of chemical sterilization composition.

Sterilization composition	S1 (73)	S2 (80)	S3 (93)
S1 (73)	0.0	7.0 <sup>ns</sup>	20.0 <sup>ns</sup>
S2 (80)	7.0 <sup>ns</sup>	0.0	13.0 <sup>ns</sup>
S3 (93)	20.0 <sup>ns</sup>	13.0 <sup>ns</sup>	0.0

( ) = Presentation of embryo germination (%)

<sup>ns</sup> = Not significantly different

**3.1.2. Black rice seed culture.** Rice seeds of black rice Toraja result of embryo culture that has germinated, will grow and develop plantlet. The germination of black rice seeds of Toraja with HM and HP color character is better than HS (figure 2). The time it takes seeds to start germinating gives a difference to the average length of the plantlet plumula on the three colors of the seed. The average length of plumula from a small black seed (HS) is 1.1 cm, medium black average length of 1.8 cm and full black average length of 1.7 cm (table 2).



**Figure 2.** The germination of black rice seeds of Toraja rice is the result of embryo culture.

**Table 2.** The average length of black rice plumula of Toraja resulting from embryo culture at 7 days after culture.

The color of embryo	HS (1.1)	HM (1.8)	HP (1.7)
HS (1.1)	0.0	0.7 <sup>ns</sup>	0.6 <sup>ns</sup>
HM (1.8)	0.7 <sup>ns</sup>	0.0	0.1 <sup>ns</sup>
HP (1.7)	0.6 <sup>ns</sup>	0.1 <sup>ns</sup>	0.0

() = The average length of plumula (cm)

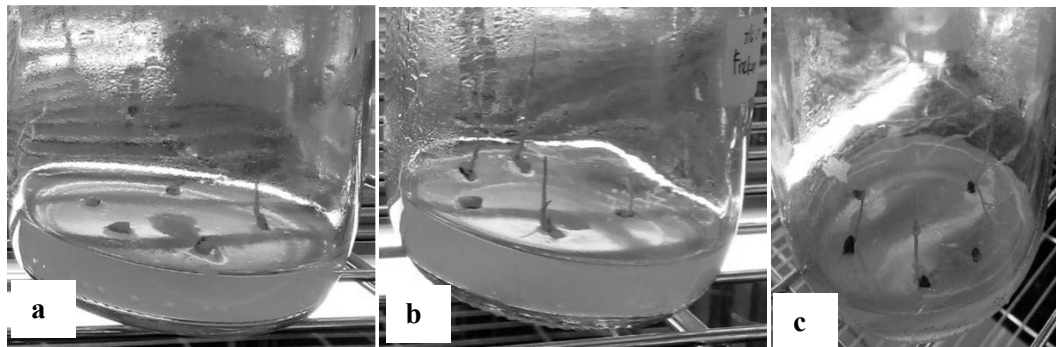
<sup>ns</sup> = Not significantly different

### 3.2. Discussion

The first experiment was conducted by determining the exact order of sterilization materials for the sterilization of the black rice embryo of Toraja. The composition of the sterilizing agent is one of the main keys in tissue culture. The materials often used for wet sterilization are alcohol and chlorox. Alcohol is a suitable sterilizing agent but also very toxic to plants. Therefore, explant is usually exposed for several seconds or minutes [8].

Chlorox compounds are commonly used as a disinfectant or bleach. This compound is very effective in killing bacteria and viruses, cleaning microorganisms that are embedded in plant material, removing soil particles, dust and others. In plant tissue culture techniques, this compound is commonly used as a sterilizing agent of plant tissue surfaces [9, 10]. The use of Chlorox as a surface sterilizing agent from various sources of plant explants has been widely reported [11, 12, 13, 14, 15, 16].

The concentration and time of using Chlorox solution per explant vary. So with the right concentration and time of exposure, because the less Chlorox concentration the explants are more susceptible to the pathogen, but if the higher concentration of Chlorox the development of tissue explant become obstructed [17]. It is seen that by using S3 composition, the color of black rice seed did not fade Unlike the sterilization using material composition S1 and S2 (figure 3).



**Figure 3.** Culture of black rice embryo with different sterilization composition. (a) Composition S1, (b) S2 Composition, and (c) S3 Composition.

The process of seed germination is a complex sequence of physiological, morphological and biochemical changes. In the case of germination of black rice seeds of Toraja, the seeds that have a darker black color (black medium and black full) more quickly experience the germination process. We suspected thickness of the aleurone, cause faster enzyme activity, resulting in the hormone to work faster. This growth hormone activity that triggers the germination of black rice seeds Toraja faster.

The concentration and duration of seed immersion in the Chlorox solution, caused the black color of the rice seed to fade. In addition, the Chlorox solution and the composition of the sterilizing agent can also cause damaged embryos, browning, unable to grow and thrive in a culture bottle and eventually die.

#### 4. Conclusion

The black rice seeds of Toraja have three color characteristics: black full (outside and inside of rice) (HP), medium black (outside and slightly inside rice) (HM), and thin black (only the outside of rice) (HS). The best sterilization for black rice embryo culture Toraja using S3 sterilization sequence (Alcohol 70% for 10 minutes and Chlorox 1% for 5 minutes). Toraja black rice rice embryo culture on MS medium has succeeded in growing a sterile plantlet, with best growth in plantlet from seeds with medium black and black color.

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