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Research Article

Optimization of *in vitro* conditions for embryo culture of *Euryale ferox* Salisb. (Makhana) using 2, 4-D as growth regulator

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Abstract: Culture conditions were optimized for high-frequency plant regulation via zygotic embryogenesis from cell culture of *Euryale ferox* (Makhana). Zygotic embryo developed into the yellowish globular structure and small callus followed by shoot formation at a frequency of 40%. When cultured on half-strength Murashige and Skoog (MS) medium, supplemented with 0, 0.1, 0.3, 0.5, 1.0, 1.5 mg/L of 2,4-D as growth regulator, the frequency of yellowish globular structure increased flabbily.

Keywords: *Euryale ferox; in vitro*; embryo culture; 2, 4-D; optimization; Makhana

Introduction

Aquatic plants are a very useful source for the recovery of ecosystem by purifying the water. *Euryale ferox* (Makhana) is a primitive dicot belonging to Nymphaeaceae. (Kumari *et al.*, 2014). It is a cash crop cultivated normally without application of any chemical fertilizers or manures. It is grown in both field and pond conditions in the stagnant fresh water bodies of north Bihar mainly in Darbhanga, Kosi and Purnea divisions (Figure 1). The plant does best in hot, dry summers and cold winters. Outside India its wild populations are available mainly in China, Japan and Korea etc. For its proper growth and development, the conducive environment is -20° to -35°C temp., 50-90% R.H, 100 cm-250 cm precipitation. The organic matter from the previous crop supports the subsequent crop. Makhana has thick fibrous roots compr-ising of 4-6 clusters, each consisting of about 15 rootlets. *E. ferox* is a perennial rooted floating macrophyte growing from a short, thick, erect rhizome. It is best raised annually from seed. Leaves show heterophyllous growth in the form of 4 different types appearing in a chronological order of sinuate, hastate, sagittate and orbicular leaves. The last set of leaves is prickly, with spines all over the plant body except roots. The fresh seeds are lumpy and surrounded by golden arils that facilitate the dehisced seeds to float on the water surface for 3-4 days and then falling to the pond bottom after flattening of the aril. The seed diameter varies from 0.5 to 1.5 cm. Kumar *et al.*, (2017) optimized the situation for germination of its seeds under polyhouse conditions. The edible part of the seed is its perisperm, which is white and starchy in nature. The plant is known for its high nutritional and therapeutic values (Jha *et al.*, 1991 a,b,; 2019).

There is no previous report of embryo culture in the case of *E.ferox.* In view of the growing need of raising the production of the crop through its introduction in waterlogged areas of other states, it is essential that tissue culture methods be applied for its easy propagation. Early attempts on lab culture of this plant have been

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unsuccessful on account of the fact that seeds are inherently contaminated with microbes that easily proliferate in the medium as well. The present experiment has for the first time optimized the *in-vitro* conditions for embryo culture of *E. ferox* using 2, 4- D as growth regulator.

Figure 1: *Euryale ferox* (Makhana) plants in field condition

Figure 2: Raw *E. ferox* seeds collected directly from the fruit.

Materials and Methods

Plant material and culture condition

Seeds of *E. ferox* collected from the Research Center for Makhana, Darbhanga, Bihar, India (Figure 2) were surface sterilized in tap water for 20 min. and thereafter were washed with teepol for 20 min. It was followed by treatment with bavistin for 10 min. Thereafter the seeds were treated with 0.5% Sodium hypochlorite solution for 10 min, followed by treatment with 0.1% Mercuric chloride ($HgCl_2$) for 5 min. and final 4 times rinsing with sterile distilled water. These surface sterilized seeds were soaked with autoclaved distilled water and then stored for 24hrs. to 48 hrs. at 25 ±2˚C in dark condition to separate the embryo from cotyledons.

Preparation of the culture medium

The embryo explants isolated from the seeds were kept on half strength MS medium consisting of 1.5% sucrose and 0.8% agar in jam bottles. Different concentrations of 2,4-D as plant growth regulator were added to half strength MS medium (Murashige & Skoog 1962) that contained macroelements - 50ml/L, microelements- 10ml/L, feEDTA-5ml/L, vitamins-1ml/L, glycine-1ml/L, sucrose-30gm/L, inositol-100gm/L and agar 8gm/L. The p^H was maintained to 5.8 with 1N NaOH before autoclaving at 121^0C at 15 psi for 21 min. Finally, 50 ml aliquots of media were dispensed in jam bottle.

Impact of the 2,4-D concentration on embroyogenic shoot formation

To the study of effect of the ratio of 2, 4-dichlorophenoxyacetic acid (2,4-D) on embryo formation from the seed of *E.ferox* surface sterilized seeds (approx. 0.5-1.0 cm diameter) were longitudinally dissected with forceps and scalpel in the laminar air flow clean bench. Zygotic embryos (approximately 1mm in length) were collected from the dissected seeds and placed onto half strength MS medium containing 0, 0.1, 0.3, 0.5, 1.0, 1.5 mg/L of 2-4, D. Each treatment consisted of two explants per jam bottle with five replicates. These cultures were maintained at 25±2˚C in the light (16 µmol m^{-2} s^{-1}). After 3 weeks of culture the regularity of explants generating pale yellow globular structure was counted for each treatment.

Results and Discussion

In this research, Culture conditions were established for high frequency plant regeneration via zygotic embryogenesis from cell culture of *E. ferox*. Zygotic embryo was cultured on half

strength MS medium containing 2,4-D. Pale yellow globular structures began to form on the surface of the zygotic embryo after 3 weeks of culture. When transferred to half strength MS basal medium, these structures developed into multiple shoots after 4 weeks of culture. The results indicate that the initial pale-yellow structure (callus) (Figure 3) developed as zygotic embryo shoots (Figure 4). Figure 5 shows its further developed stage.

Figure 3: Callus developed from the embryo of *E. ferox*.

Figure 4: Shoot developed from the callus

It was found that there was no significant difference in the number of shoots developing from 0.1-0.5 mg/L concentration of 2,4-D. At concentration 1.0 mg/L the maximum number of shoots (40%) was observed. On increasing the concentration to 1.5 mg/L of 2,4-D the number of shoots decreased. (Figure 6)

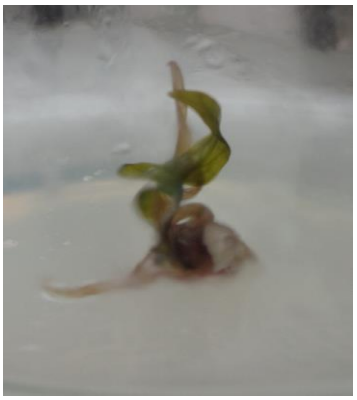

Figure 5: Further differentiation of the shoot.

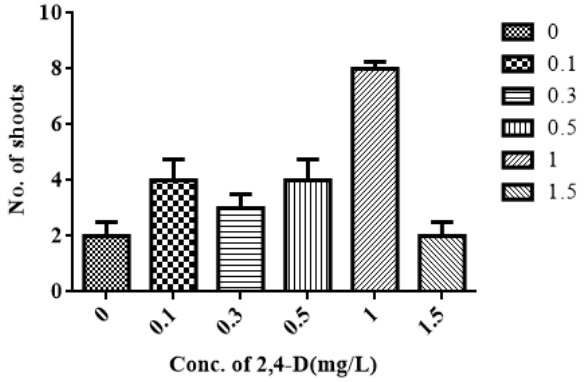

Figure 6: Figure showing relationship between concentrations of 2, 4-D and the number of shoots

Conclusion

The present research met its objective by developing an efficient *in-vitro* high frequency plant embryo culture system for the aquatic plant *E. ferox.* that has a high potential for future environmental studies and can be used in phytoremediation of polluted water bodies contaminated with heavy metals (Rai *et al.,* 2002). Easy propagation of the plant could help retrieve other parts of the plant like seed coat which is being exploited for therapeutic purposes (Jian *et al.,* 2019). There is a need to mitigate imminent climate change through enhancement of phytoaquatic productivity adopting improved technology is for mass production of plants like *E. ferox*. The embryo culture protocol needs to be further modified after regeneration of its full plants.

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