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High Efficiency *In vitro* Whole Plant Regeneration via Desiccated Callus in *Oryza sativa* cv. MTU1010

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Abstract:- For most people across the planet, rice is also considered as most beneficial crops in terms of fulfilling every day's energy and nutritional needs. Worldwide food safety is seriously threatened since severe limitations on rice output caused by both abiotic and biotic variables. Even though rice features much more developed regeneration mechanism than other agricultural crops, majority of desi cultivars continue to stay as impervious to genetic alterations and regeneration. Therefore, among preliminary steps in the transgenic plant generation necessitates to improve tissue culture procedures to produce viable plants via embryogenic calli mediated regeneration. In this study, we reporting that enhanced rate of plant regeneration was observed from mature seed-derived embryogenic calli of the indica rice cultivar MTU1010 following partially desiccation procedure. We studied the effects of several plant growth regulators on the efficiency of inducing embryogenic callus and regeneration of whole plantlets. The MS-CIM medium with 2.0 mg-1 2,4, D showed the highest callus induction percentage. Maximum rate of shoot regeneration (92%) was observed from 48hr partially dehydrated calli, when enriched with high cytokinin (2 mg/l kinetin) and lower auxin (0.5 mg/l NAA) concentrations. Calli that had been moderately dehydrated showed significantly greater regeneration rates than undehydrated calli. Upon being transferred onto a rooting medium, the well-rooted plantlets were shifted to shaded glass house for hardening. Hence, this approach is rather simple to apply and can be employed in genetic modification studies alongside other biotechnological endeavours.

Keywords:- *Rice, MTU1010, Desiccation, Callus Induction, In vitro Regeneration.*

I. INTRODUCTION

To ensure that 852 million metric tons of paddy are produced by 2035, the growing population requires an enhanced and sustainable strategy of rice agriculture using more productive cultivars [1]. Numerous biotic and abiotic factors can hinder rice productivity. Two important abiotic aspects that significantly reduce the yield of paddy worldwide are salt and drought [2]. The usual breeding approach is insufficient to boost rice productivity and harvests Since there is a lack of genetic variety [3]. The limited genetic base and sexual incompatibility of standard breeding to create tolerant lines have led way to a promising alternative via genetic engineering. To boost rice yield, a range of ancient and contemporary techniques are being used, including breeding, genetic engineering, and genome editing. It is possible to reproduce elite rice varieties that can withstand harsh climate conditions by integrating traditional and innovative genetic modification techniques with in vitro plant tissue culture techniques [4]. Plant regeneration through in vitro method is a crucial and fundamental part of biotechnology, which is needed to manipulate plant genetics. High-fidelity regeneration from in-vitro cultivated cells or tissues are a prerequisite for agricultural scientists to effectively exploit tissue culture and genetic engineering technology. In order to raise the probability of plant revival, numerous factors are being studied in rice. Before transferring callus onto regeneration medium, they are partly desiccated which enhanced regenerative process in callus cultures [5].

A prominent semi-dwarf variety with long, thin grains that can withstand blast disease and BPH, respectively, is Cotton Dora Sannalu (MTU 1010). It is frequently employed to be parent in many trait improvement breeding efforts and typically grows in subtropical and tropical agroclimatic zones.

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Nearly six percent out of nation's gross rice cultivating area is devoted to MTU1010, that significantly contributes towards the nation's food supply and generates income ranging from Rs. 1200 to Rs. 1500 crores annually [6]. Relatively little study was done to improve the effectiveness of regeneration in Oryza sativa indica genotypes, although knowing that numerous procedures for regeneration have already been accomplished and standardized in japonica cultivars. By adjusting several parameters in tissue culture techniques, the current study seeks to develop a fast, repeatable, effective, and optimal way for MTU1010, with the goal of improving the induction of callus and encouraging regeneration by means of dehydrated callus.

➢ *Abbreviations*

MS	Murashige and Skoog medium
CIM	Callus induction medium
SIM	Shoot induction medium
BAP	6-Benzylaminopurine
NAA	1-Naphthaleneacetic acid
2,4-D	2,4-Dichlorophenoxyacetic acid
Hgcl2	Mercuric chloride

II. MATERIALS AND METHODS

➢ *Plant Material and Explant*

Oryza sativa MTU 1010, referred as Cotton Dora Sannalu, a remarkable, high-yielding, extensively farmed mega-variety featuring long grain type that was developed and published by Acharya NG Ranga Agricultural University (ANGRAU), India. MTU 1010 was picked for present study. The germplasm was received from Indian Institute of Rice Research (IIRR), Hyderabad and maintained at green house of Centre for Plant Molecular Biology (CPMB), Osmania University. Viable seeds of MTU1010 were dehusked carefully evading the damage of embryo. Subsequently, Healthy good-looking seeds were chosen.

➢ *Media Preparation*

Murashigue and Skoog (MS) medium with multiple levels of hormones were used. Regarding callus induction MS-Callus induction media (CIM) components were tabulated (Table 1). To induce callus, various concentrations of auxin (2,4-D) were employed. All the components were put in 1000ml conical flask, mixed well, pH was set to 5.6, parted into 200ml each. Then 0.3% plant gel rite is mixed to media, autoclaved for 15-20 minutes. After autoclaving the medium was permitted to recede 30^0C, and Then inside the laminar flow cabinet each medium flask is supplemented with varying concentrations of hormones. Hormones were filter sterilised before adding to media. Similarly, shooting, and rooting medium were prepared with desired hormonal concentrations. A concentration of 2% Gel rite was opted for rooting. The details of hormonal combinations were tabulated. (Table 4).

➢ *Surface Sterilisation of Explant*

The mature dehusked seeds were surface sterilised by rinse with distilled water for about 2-3 times. These seeds were collected into a clean autoclaved falcon tube, followed by addition of surface detergent – TWEEN20 and rinsed for 5-10 mins and washed thoroughly with water until foam is removed. Further steps are performed inside the flow cabinet. The seeds were sterilized with sodium hypochlorite (commercial bleach) for 2-5 minutes and subsequently washed twice with autoclaved distilled water. 1:1 w/v Bavistin solution (commercial fungicide) was added, washed for about 5 minutes, and cleansed several times with sterile distilled water. Thereby subjected to 70% ethanol for approximately 30-35 seconds and immediately washed using distilled water. At last, washed using 0.1% Hgcl2 solution for 5-7 minutes with constant manual shaking. Thorough washing of seeds for 3-4 times by autoclaved water was done. Placed them on blotting paper and kept aside to dry completely within flow cabinet. They were collected into clean falcon tube and sealed using parafilm for further.

III. SEED INOCULATION AND INDUCTION OF CALLUS

The inoculation of completely dried, undamaged, healthy seeds was conducted within laminar flow of culture room facility of Centre for Plant Molecular Biology, Osmania University. Prior to the process the laminar flow unit is sterilized by subjecting to UV-light lasting 10 to 20 minutes. To avoid contamination hands were cleansed and sanitized with absolute spirit while platform of laminar unit is wiped using spirit. Autoclaved and Sterile Petri dishes, forceps, CIM with different hormonal concentrations were placed on flow platform subjected to UV radiation for 5-10 minutes. Then inoculate the sterile seeds over media filled petriplates with a pair of sterile forceps ensuring that seeds are placed as embryo part facing the media, pressed gently but not embedded. Tools were dipped in 75% ethanol after each inoculation to reduce the chances of contamination. After inoculation is done, the plates were wrapped by parafilm, properly labelled. Incubate these plates under dark conditions for 7-10 days at 22^0C-28^0C. Using the formula presented below, the callus induction % was determined [7].

$$\text{Callus induction frequency}(\%) = \frac{\text{No. of seeds producing calli}}{\text{No. of seeds cultured}} \times 100$$

➢ *Subculture of Embryogenic Callus*

After 7 days, embryo from inoculated seeds showed signs of callus initiation along with germination of them. About 8-11 days old callus needs to be carefully cut excluding the sprouting shoot with sterile forceps and scalpel. Now these are replaced onto callus subculture media (same as CIM). After a week incubation, callus starts proliferating and increases in mass. These are again sub cultured carefully onto freshly prepared CIM medium containing petri dishes without damaging the callus. Incubate in dark conditions for 6-8 days at 22^0C -25^0C.

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➢ *Partial Desiccation*

One month old calli were placed on sterilized petri plates with two sterile Whatman1 filter papers within to achieve the desired dehydration level. Then the culture plates were enclosed by parafilm and incubated at dark conditions at 25±1°C for 48 hours. The partly dehydrated calli were shifted on to regeneration media with varied concentrations of growth regulators [8].

➢ *Regeneration*

- *Shoot Formation from Desiccated Callus.*

After 8-10 days, proliferated callus mass is transferred into bottles with shooting media (Table 2) one containing 3.0mg/l BAP+ 1.0mg/l Kinetin+ 0.5mg/l NAA; and another containing kinetin@2.0 mg/l + NAA@0.5 mg/l; subsequently placed in light for 14–16 hours, then in the dark lasting 10–15 days with intensity threshold of 2000 lux at 20-25C temperature. When the callus mass starts to show green, they are sub cultured to same shooting media respectively for further proliferation. The formula below determines regeneration frequency [7].

$$\text{Plant regeneration}(\%) = \frac{\text{No. of calli producing plants}}{\text{No. of plants planted}} \times 100$$

- *Rooting and Acclimatization.*

When the growing leaflets are observed, they are separated from callus and are positioned upright in flasks comprising rooting medium (Fig 2C). Half MS-medium without supplements and another media containing 0.05mg/l NAA with 2% Gel rite was used to facilitate root growth (Table 3). Flasks were incubated under 14-16hrs light and 6 hrs dark until roots are formed firmly. Well-rooted shoots were gently removed from the culture flasks, properly rinsed over running tap water to eliminate any remnants of media, and placed into tiny nursery pots filled with a sterile 1:1 vermicompost and soil and covered using polytene bags with holes and moved to glass house (Fig 2D). Subsequently, plants were introduced to open air after eight to ten days. In the end, initially hardened plantlets have been moved to plastic pots filled with black soil, making sure not to disrupt their roots (Fig 2E). They were afterwards permitted to mature under controlled conditions inside the shaded glass house. (Fig 2F).

IV. RESULTS AND DISCUSSION

The main purpose of this investigation was to develop a successful regeneration strategy with high rate via desiccation of callus for MTU1010 Indica rice variety. The findings of this experiment revealed the high frequency invitro regeneration ability of the cultivar MTU1010 through desiccation. To find the precise process for explant sterilization, callogenesis and regeneration for this cultivar is the main objective of research. Two phases are comprised in the entire execution. During the preliminary stage, callus induction was performed for specifically chosen rice type at different concentration levels of auxin provided [9]. The responses of callus induction against MS media with various concentrations of growth regulators were recorded. The following phase involved plant regeneration through desiccation of callus. The impact of various hormone additions on MS media on in vitro plant regeneration was ascertained.

In the current study, desiccated callus was used as tool to explore *in vitro* plant regeneration. To minimize the probability of contamination, the explants (mature embryo) were surface sterilized by using various sterilizing agents. The development and growth of callus was done on MS medium enriched with varying amounts of 2, 4-D. After 8-11 days from inoculation of dehusked seeds on CIM1 media, shown germination and induction of callus simultaneously. With reference to Fig 1B, 7 days after inoculation in CIM media, seeds showed sprouting with callus. Maximum induction of calluses has achieved with an amount of 2.0 mg/l of 2, 4-D (Fig 1B) which corelates with previous findings of Libin, A., et al. (2012) [10]. About 96% of callus was formed when mature embryos are incubated on MS medium containing 2,4-D (2.0mg/l) and (2.5mg/l). All the callus masses had a creamy tint, friable texture and are embryogenic (Fig 1D). The 0.5 mg/l 2, 4-D MS medium was unable to trigger the callus growth (Fig 2A). It turned out that whereas 2,4-D at lower doses permitted morphogenesis, higher quantities inhibited callus proliferation. It was found that 2.0 mg/L of 2,4-D was ideal level for sufficient inducement of callus and its multiplication [11], [12]. This also applied to following subcultures.

The conclusions from this research suggest that MTU1010 has a greater capability to develop callus and appears bulkier because of obvious actively dividing cells when augmented with CIM media. The varying hormonal concentrations influence the growth of callus. Shweta, S., et al. 2020's earlier reports are related with current findings. The callus was subcultured for two rounds after eight days each for development and maintenance [13].

Callus turns slightly yellowish in colour after dehydration treatment (Fig 1E). Partially desiccated calli increased the rate of regeneration process [8]. After partial desiccation, an advanced regeneration rate of 93% was noted in MS medium with the hormone concentration of 2.0 mg/l kinetin+0.5 mg/l NAA, in contrast to merely 54% in without the use. A hormone concentration of 3.0 mg/l BAP+0.5 mg/l NAA along with small quantity of kinetin@1.0mg/l, an enhanced regeneration rate of 56% was seen following partial desiccation, in contrast to just 32% in without desiccated ones. Callus on medium with BAP showed multiple shoots.

Previous reports of Behera et al. (2019); Sahoo et al (2011); [14], [15] suggested that the elevated regeneration frequency occurred at 3.0mg/l BAP+ 1.0mg/l Kinetin+ 0.5mg/l NAA in some indica varieties (IR64, Swarna) whereas 2.0mg/l kinetin+0.5mg/l NAA in other indica varieties (PB1 and CSR10). However, both combinations were employed in present study. An increased regeneration rate of 86% was noted in cytokinin and auxin at

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a combination of 2.0 mg/l kinetin plus 0.5 mg/l NAA. Approximately five to eight days were needed to promote callus development and regeneration. Greenish appearance was identified after 7 days of being subjected to shooting media as shown in figure 1F in two hormonal combinations. Additionally, with same medium, shoot growth, multiplication, and appropriate development were noted (Fig 2B). At recurring times of 10-14 days, they were sub cultured over same media for their proliferation and growth.

In half MS, all the proliferated shoots (99%) shown root development with no hormonal supplements [16]. Using of 0.05mg/l NAA+ ½ strength MS medium showed earlier root formation. Gel-rite (2%) served as gelling agent for rooting medium [17].

V. CONCLUSION

In the current research, we reporting that high rate of regeneration (96%) was achieved from desiccated calli of rice cultivar MTU1010, a frequently employed variety in various fields of Genetic engineering and Plant Biotechnology research for trait improvement. Various combinations of callus induction and regeneration media with different concentrations of growth hormones were employed for in vitro regeneration of whole plant. MS-medium with 2.0 mg-1 2,4, D facilitated highest callus growth**.** Maximum rate of shoot regeneration (92%) was observed from partially dehydrated calli on MS medium augmented with 2 mg/l kinetin + 0.5 mg/l NAA. This approach is comparatively simple to apply and may be employed for genetic transformation research as well as other biotechnological endeavours in the future, moreover, this protocol will aid to preserve the rice variety.

TABLES

Table 1- Components of Callus Induction Medium.

MS - Callus induction medium (CIM) per Litre - pH 5.6	
MS salts	4.1g
Maltose	30g
Inositol	100mg
Casein hydrolysate	200mg
Tryptophan	50mg
Nicotinic acid	0.5mg
Pyridine.Hcl	0.5mg
Thymidine.Hcl	1mg
Glycine	2mg
Gelrite	3g

Table 2- Components of Shooting Medium.

MS- Shoot induction medium (SIM) per Litre -pH 5.8	
Ms salts	4.1g
Maltose	30g
Inositol	100mg
Glycine	2 mg
Thymidine. Hcl	1mg
Nicotinic acid	0.5mg
Pyridine.Hcl	0.5mg
Gelrite	3g

Table 3- Components of Rooting Medium.

MS- Rooting medium per Litre - pH 5.8	
Ms salts	2.0g
Maltose	36g
Nicotinic acid	0.25mg
Pyridine.Hcl	0.25mg
Thymidine.Hcl	0.5mg
Glycine	1mg
Gelrite	2 g

Table 4- Combinations of Hormonal Concentrations Used.

MEDIUM	Hormone Combinations and Concentrations
MS-CIM	2,4-D@ 0.5mg/l
MS-CIM	2,4-D@ 1.0mg/l
MS-CIM	2,4-D@ 1.5mg/l
MS-CIM	2,4-D@ 2.0mg/l
MS-CIM	2,4-D@ 2.5mg/l
MS-SIM	3.0mg/l BAP+ 1.0mg/l Kinetin+ 0.5mg/l NAA
MS-SIM	Kinetin @2.0mg/l +NAA@0.5mg/l
MS- Rooting medium	No hormones
MS- Rooting medium	0.05mg/l NAA

Table 5- Callus induction % in CIM with Different Levels.

Hormone Concentration	Number of Explants	Number of Calli	Callus Induction %
2,4-D@0.5mg/l	100	0	0%
2,4-D@1.0mg/l	100	34	34%
2,4-D@1.5mg/l	100	53	53%
2,4-D@2.0mg/l	100	96	96%
2, 4-D@2.5mg/l	100	94	94%

Table 6- Regeneration% with Relation to Hormonal Concentrations.

Hormone Concentration	Desiccation	Number of Calli	Number of Shoots	Regeneration%
3.0mg/l BAP+ 1.0mg/l Kinetin+ 0.5mg/l NAA	Yes	50	28	56%
3.0mg/l BAP+ 1.0mg/l Kinetin+ 0.5mg/l NAA	No	50	16	32%
2.0mg/l Kinetin +0.5mg/l NAA	Yes	50	47	94%
2.0mg/l Kinetin +0.5mg/l NAA	No	50	27	54%

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FIGURES

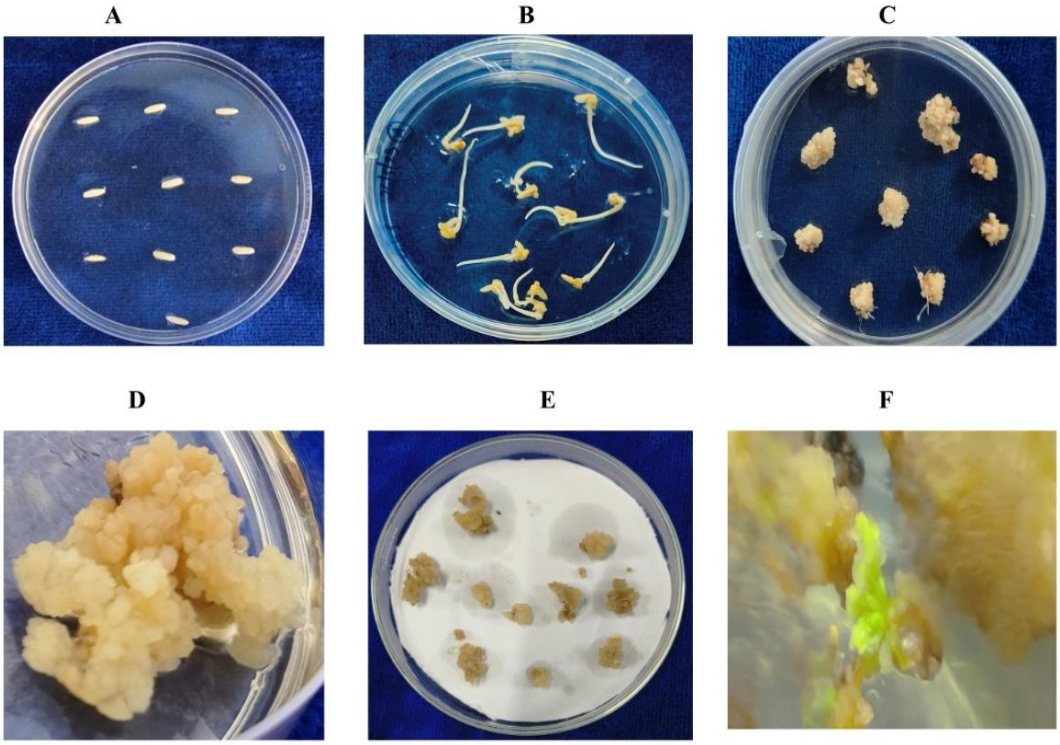

Fig 1 – A) Inoculation of Seeds onto CIM, B) Induction of Callus in CIM-2.0mg/l Medium, C) Subculture of Calluses, D) Fragile Texture and Creamy Embryogenic Callus, E) Partial Desiccation of Calli using Filter paper, F) Shoot Initiation or Greenish Appearance in Callus.

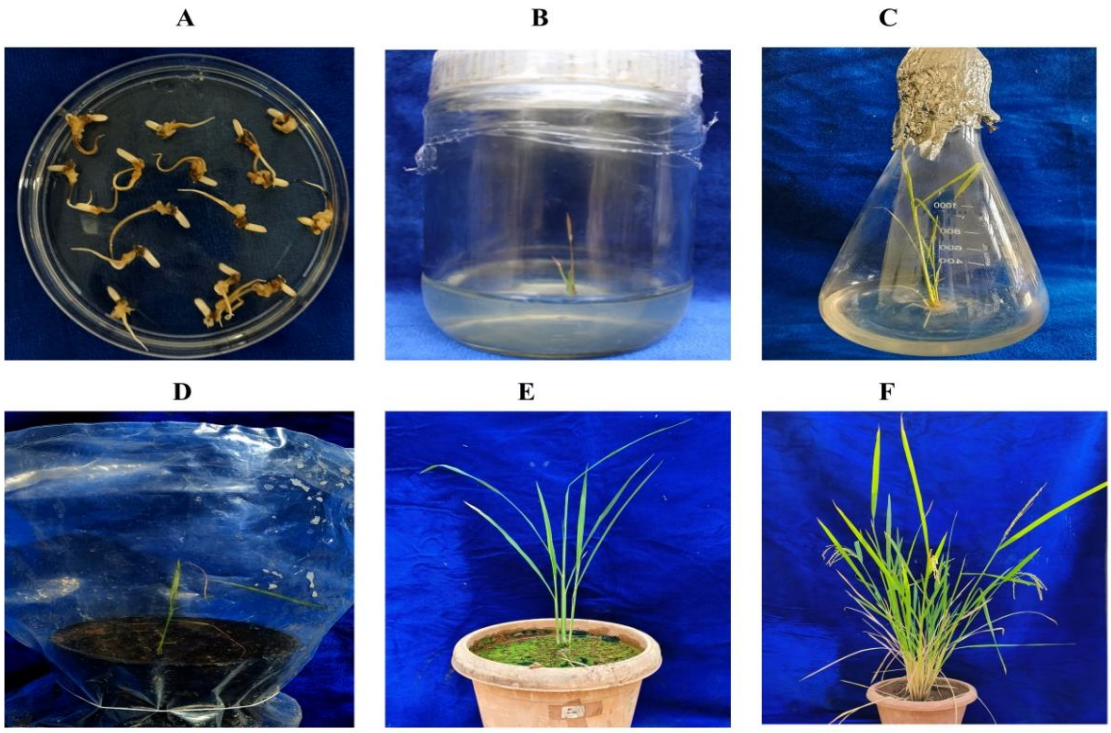

Fig 2 – A) Inoculation of Seeds onto CIM with 0.5mg/l 2,4-D, B) Subculture of Regenerated Shoot onto SIM-Kinetin+NAA Medium, C) Incubating for Root Formation, D) Acclimatisation of Plantlet, E) Plant Exposed to Environment, F) Fully Grown invitro Regenerated Plant.

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AUTHOR CONTRIBUTION STATEMENT

Anjana Priyadarshani. K, Prashant. S and Srinivas Naik. K has designed the experiments and the structure of the article and prepared the first draft. Prashanth. B have added lateral text in the manuscript and refined it. Srinivas Naik. K, Prashant. S, Prashanth. B, Anjana Priyadarshani. K, Anjana. W, Sriya Reddy. P have revised the manuscript. All authors have approved it.

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