Effect of media and photoperiod on embryogenic and organogenic callus induction from mature seed in *Oryza sativa* L.

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ABSTRACT

Rice is the staple food for billions of people and a source of income for hundreds of millions. This food security crop production needs to be improved to face the projected demands of the exponentially augmenting world's population and overcome biotic and abiotic stress in the fields. Optimized *in vitro* culture protocols are a prerequisite to genetic engineering which appears to be the best potential way to improve the rice plant. In this study, 'Nerica 3' and 'Nerica L36' seeds were *in vitro* culture tested into 4 media (m1-4), in dark and photoperiod environments. The percentage of callus induction was calculated and callus weight was recorded. Results show that callus induction was influenced by variety × environment and medium × environment interactions, with a strong influence of the environment used. 'Nerica 3' showed the highest mean (88.25%) callus induction after six weeks of incubation on different media. m1 and m2 media showed greater mean callus induction (more than 86%). Higher callus induction came from m1 (82% and 91%) and m2 (81% and 93%) media, while lower rates came from m3 (73%) and m4 (69%) mediafor embryogenic and organogeniccalli respectively. Culturing the explant in dark environment to produce embryogenic callus, resulted in greater callus weight for 'Nerica 3' (1.03 g) and 'Nerica L36' (0.79 g). This study is a contribution to the rice plant genetic improvement by proposing protocols for somatic embryogenesis of two Nerica rice varieties.

Keywords: Rice; food security; biotechnology; somatic embryogenesis; callus.

1. INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important crops and the staple food for more than half of the world population. This cereal is also a source of incomes for millions of people who directly depend on the rice value chain [1,2]. Rice is classified in many countries as a food security crop and is the object of several governmental strategies to combat hunger [3]. The world population growth demands an increase of the agricultural production in order to meet the food security goals projected by the United Nations [4]. However, the strategic crop faces production constraints which are biotic (pests and diseases) and abiotic (environment, climate), responsible of bad quantitative and qualitative yields.

Despite the introduction of improved varieties developed by conventional breeding methods, those problems have not been solved. Biotechnology techniques, and especially genetic engineering, could be more efficient ways to develop new seeds with interesting characters which can cope with the needs of producers and be more nutritive [5,6]. Genetic manipulations of grains require a subsequent protocol of somatic embryogenesis, the principal way to go through for gene material to be transferred into embryos then develop a new organism. This means to develop or find culture media with suitable genotypes responsive on *in vitro* plant regeneration[7,8]. Moreover, studies on *in vitro* plantlet

regeneration in rice using mature seed as explant are lacking although mature seed has the advantage of being available throughout the year, easy to handle and in bulk quantities.

The objective of this study was to define media and environment suitable for somatic embryogenesis of rice cultivars using mature seed as explants. While doing so, we compare the effects of media, varieties, environment and their interaction on callus induction and callus weight obtained from mature seed as explants.

2. MATERIAL AND METHODS

2.1 Plant Materials and Explants Preparation

Field grown seeds (matured caryopses) of rice cultivars 'Nerica 3', 'Nerica L36', were used as the source for mature seed culture. Nerica (New Rice for Africa) are rice varieties developed by the interspecific crossing of the Asian rice (*Oryza sativa*) and the African rice (*O. glaberrima*) by the Africa Rice Center (AfricaRice), a pan-African Center of Excellence for rice research, development and capacity building [9]

The seeds were procured from Garoua Multipurpose Research Station of Institute of Agricultural Research for Development (IRAD), Sanguéré - Paul, Cameroon.
The seeds were then surface sterilized by washing in ethanol 70% (v/v) for 3 minutes, followed by a bath of 100% commercial bleach plus a drop of Tween 20 for 30 minutes with agitation. Thereafter, they were rinsed four times in sterile distilled water.

2.2 Callus Induction and Maintenance

The disinfected seeds were aseptically placed in a Petri dish containing the induction and maintenance media based on MS basal medium (Murashige and Skoog, 1962): m1, m2, m3 and m4 (Table 1). The embryos were incubated on the media at 27°C for six weeks under a dark environment[10] and under a 16 h photoperiod environment[11] to obtain respectively embryogenic callus and organogenic callus. Subculture was performed at 21 days at the same conditions. At the end of the callogenesis, callus weight was recorded, and the percentage of callus induction was calculated as follows: (the number of callus obtained / the number of seed transferred to the induction medium) × 100.

2.3 Experimental Design and Statistical Analysis

A randomized complete block design (RCBD) was used with 2 varieties, 4 media and 2 environments (2 x 4 x 2 = 16 treatments). The treatments consisted of 5 replications of each medium for each variety and for each environment, each replication with 10 seeds. For the analysis of callus induction and callus weight, Analysis of Variance (ANOVA) was performed using the General Linear Model (GLM) procedure in SAS 9.3 software. ANOVA results were considered significant at $p < 0.05$ and means of treatments were compared using t Tests.

3. RESULTS

3.1 Callus Description

Callus initiation started four days after incubation of mature seeds as explants on all induction media in both environments in both rice varieties (Fig. 1). At twenty-one days after incubation of explants, embryogenic and organogeniccalli appeared to have the same texture at this stage of development (Fig. 1). At the end of the callogenesis, six weeks after

incubation of the explants, in variety ‘Nerica 3’, the embryogenic and organogeniccalli were characterized by a friable, granulated and smooth texture and a whitish to cream color. In the variety ‘Nerica L36’, necrotic areas and roots were also observed on the calli (Fig. 1).

3.2 Callus Induction

Callus induction was influenced not only by variety, medium and environment but also by variety × environment and medium × environment interactions (Table 2). Of the two varieties tested, ‘Nerica 3’ (88.25%) showed the highest mean callus induction after six weeks of incubation of the explants on different media (Table 3). Among the four media, callus from m1 and m2 media showed greater mean callus induction (more than 86%), whereas m4 medium showed the lowest (73.5 %; Table 3). Organogenic callus from photoperiod environment (85.5%) showed the highest mean callus induction (Table 3). However, the two varieties showed callus induction upon culturing the explants on the two environments, but at different levels (Fig. 2). In ‘Nerica 3’, higher callus induction was observed for embryogenic callus from dark environment (90%). For ‘Nerica L36’, the best callus induction came from organogenic callus from photoperiod environment (84.5%). The same is true for the interaction medium × environment (Fig. 2). Higher callus induction came from m1 (82% and 91%) and m2 (81% and 93%) media, and lower rates came from m3 (73%) and m4 (69%) media, for embryogenic and organogeniccalli respectively.

3.3 Callus Weight

Callus production was strongly influenced by the environment used (Table 2). A significant (*P< .001*) interaction between variety and environment was observed (Table 2). Callus weight measured after 6weeks of culture of the two varieties on different induction and maintenance media showed that the highest callus weight was observed for embryogenic callus on dark environment (0.91 g; Table 3). Culturing the explant on dark environment to produce embryogenic callus, resulted in greater callus weight for ‘Nerica 3’ (1.03 g) and ‘Nerica L36’ (0.79 g) whereas the production of organogenic callus in photoperiod indicated the lowest callus weight for ‘Nerica 3’ (0.77 g) and ‘Nerica L36’ (0.46 g; Fig. 3).

4. DISCUSSION

Adapted protocols, including cultivars and appropriated media, are needed to enable genetic engineering of rice. Thus, ‘Nerica 3’ and ‘Nerica L36’ seeds were *in vitro* culture tested into 4 media, in the dark and in photoperiod environments for each variety.

Callus were induced at a rate of over 70% from ‘Nerica 3’ and ‘Nerica L36’ in all experimental situations even though the first cultivar gave the higher callus induction. This means that these two varieties are highly reactive to dedifferentiation. The percentage of 90% calli induction obtained in dark incubation is close to the 96 % obtained in the same environment by[12]on Faro 55, a Nerica cultivar used in Nigeria. Furthermore, these varieties were successfully used in the creation of genetically modified rice through *Agrobacteriumtumefaciens* infection [13]. These plant materials could provide an interesting tool for genetic manipulations with this ability to express a high ratio of calli in molecular biotechnology protocols.

‘Nerica L36’ seems the be more efficient in expressing organogeniccalli than embryogenic. Early roots apparition with callus expression may signify that the 2 physiologic pathways are closely related. This fact tend to corroborate the hypothesis made by Zhang et al. [14], stating that callus formation occurs through the root development pathway.

Media m1 and m2 showed the calli induction higher than media m3 and m4 for the two varieties. The plant reaction to regeneration depends on the composition of the culture media[11]. MS vitamins present in m1 and m2 may be more efficient than B5 vitamins present in m3 and m4 media, concerning callus dedifferentiation. The difference in the content of some vitamins Nicotinic Acid, Pyridoxine-HCl, Thiamine-HCl, and Glycine (present only in the MS basal and absent in B5). The latter could participate in a better development of calli in m1 and m2 media.However, in eggplant (*Solanum melongena* L.), the results exhibited the significant role of the B5 medium in improving microspore androgenesis and suggested that this medium can be a new way to progress microspore-derived callus formation culture in this plant than MS and modified NLN media[15].

Although photoperiod environment induced more calli than dark environment, the highest callus weight was obtained by embryogenic calli.However, in *Basella rubra* L. (an important green leafy vegetable vine), Kumar et al. [16] recorded that, the growth curve recorded for 6 weeks of culturing revealed that the photoperiod effect was found to be pivotal for acquiring biomass: the continuous light supported maximum biomass (12.42 g) production followed by the 16:8 h photoperiod (9.02 g) and continuous darkness (4.28 g). In addition, the analysis of variancesin this study indicates a strong relationship between the variety and the growing environment for callus induction and callus weight. Incubation in the dark would be the best option for a better quantity of calli production for 'Nerica 3' and 'Nerica L36' seed explant cultivars. However, Pathi et al. [17]had showed that, in regeneration stage, the regenerated callus yielded higher number of shoots in both embryogenic andorganogeniccalli from maize mature seed as explant.

5. CONCLUSION

In this study, we identified for the first time, favourable media and environment for callus inductionfrom mature seed of two Nerica rice varieties. These mediawill be used for embryogenic callus induction from mature seed in the rice plant genetic improvement program.

REFERENCES

1 Bandumula N. Rice Production in Asia: Key to Global Food Security. Proc Natl Acad Sci, India, Sect B Biol Sci. 2018;88:1323–1328.

2 Zibaee A. Rice: Importance and Future. J Rice Res. 2013;1(e102).

3 FAO. Rice Market Monitor. Food and Agriculture Organization of the United Nations VOLUME XXI ISSUE No. 1April 2018;2018:1-38. Accessed 13 September 2023.Available: https://www.fao.org/3/I9243EN/i9243en.pdf.

4 United Nations. Transforming our world: The 2030 Agenda for Sustainable Development. 2016;1-41.Accessed 13 September 2023. Available: https://wedocs.unep.org/20.500.11822/11125.

5 Datta S, Bouis HE. Application of Biotechnology to Improving the Nutritional Quality of Rice. Food Nutr Bull. 2000;21:451–456.

6 Datta SK. Rice Biotechnology: A Need for Developing Countries. AgBioForum. 2004;7:31–35.

7 Ghobeishavi, H, Ebrahim D, Alavikia SS, et al. Study of Factors Influencing Somatic Embryogenesis in Rice(Oryza Sativa L.). International Journal of Advanced Biological Research. 2015;3:43–50.

8 Kumar Sah S, Kaur A. High Frequency Embryogenic Callus Induction and Whole Plant Regeneration in Japonica Rice Cv. Kitaake. J Rice Res. 2014;2(125).

9 Mohapatra S.Nerica – Tailor-Made Innovation for Africa's Rainfed Rice Ecology. Rural 21. 2019;13-15. Accessed 13 September 2023. Available: https://www.africarice.org/_files/ugd/0839e4_7bb926daca244e8588de259f3d233f4c.pdf.
10 Mohd Din ARJ, Iliyas Ahmad F, Wagiran A, et al. Improvement of efficient *in vitro* regeneration potential of mature callus induced from Malaysian upland rice seed (*Oryza sativa* cv. Panderas). Saudi J Biol Sci. 2016;23:S69–S77.
11 Pawar B, Kale P, Bahurupe J, et al. Proline and Glutamine Improve *in vitro* Callus Induction and Subsequent Shooting in Rice. Rice Sci. 2015;22:283–289.
12 Afolabi A, Oyebanji OV, Odusanya O s, et al. Regeneration of plants from rice caryopsis derived callus culture of Nigerian local cv. Suakoko 8 and a NERICA cv. FARO 55. Afr J Plant Sci. 2008;2:109–112.
13 Ishizaki T, Kumashiro T. Genetic transformation of NERICA, interspecific hybrid rice between Oryza glaberrima and O. sativa, mediated by Agrobacterium tumefaciens. Plant Cell Rep. 2008;27:319–327.
14 Zhang Z, Zhao H, Li W, et al. Genome-wide association study of callus induction variation to explore the callus formation mechanism of rice. J Integr Plant Biol. 2019;61:1134–1150.
15 Hashemi M, Moieni A, Sabet MS et al. Introducing Gamborg's B5, a high-potential medium for isolated microspore culture, and presenting a new MS medium-based protocol for androgenic plant regeneration in eggplant (*Solanum melongena* L.). Plant Cell Tiss Organ Cult. 2024;156(79).
16 Kumar SS, Arya M, Mahadevappa P, Giridhar P. Influence of photoperiod on growth, bioactive compounds and antioxidant activity in callus cultures of *Basella rubra* L. J Photochem Photobiol B: Biol. 2020;209:111937.
17 Pathi KM, Tula S, Huda KMdK, et al. An efficient and rapid regeneration via multiple shoot induction from mature seed derived embryogenic and organogenic callus of Indian maize (Zea mays L.). Plant Signal Behav. 2013;8:e25891.
18 Gamborg OL, Miller RA, Ojima K. Nutrient requirements of suspension cultures of soybean root cells. Exp Cell Res. 1968;50:151–158.
19 Murashige T, Skoog F. A Revised Medium for Rapid Growth and Bio Assays with Tobacco Tissue Cultures. Physiologia Plantarum. 2006;15:473–497.

TABLES

Table 1. Media composition

	Media tested			
Components	**m1**	**m2**	**m3**	**m4**
Macroelements	MS	MS	MS	MS
Oligoelements	MS	MS	MS	MS
Vitamins	MS	MS	B5	B5
Fe-EDTA	MS	MS	MS	MS
Myo-Inositol (mg/l)	100	100	100	100
Sucrose (g/l)	30	30	30	30
2,4-D (mg/l)	2	2	2	2
BA (mg/l)	0	1	0	1
pH	5,7- 5.8	5,7- 5.8	5,7- 5,8	5,7- 5,8
agar (g/l)	8	8	8	8

2,4-D: 2,4 Dichlorophenoxyacetic acid; BA: 6-benzylaminopurine; B5: Gamborg B5 medium[18] ; MS: Murashige and Skoog medium[19].

Table 2. Analysis of variance for effects of variety, medium, environment and their interactions on callus induction (CI) and on callus weight (CW) in rice.

Source	F value		
	DF	CI (%)	CW (g)
Variety (V)	1	39.37**	0.67
Medium (M)	3	9.95**	0.55
Environment (E)	1	12.35*	45.61**
V x M	3	2.71	0.53
V x E	1	27.78**	39.39**
M x E	3	7.69*	1.89
V x M x E	3	1.87	2.47

* Significant at *P* < *.01*; ** Significant at *P* < *.001*

Table 3. Mean of callus induction (CI) and of callus weight (CW) of two rice varieties obtained on four induction and maintenance media after 6 weeks of culturing in two environments.

	CI (%)	CW (g)
Variety		
Nerica 3	88.25[a]	0.74[a]
Nerica L36	75.75[b]	0.78[a]
LSD	3.97	0.08
Medium		
M1	86.5[ab]	0.72[a]
M2	87[a]	0.75[a]
M3	81[b]	0.78[a]
M4	73.5[c]	0.80[a]
LSD	5.62	0.12
Environment		
Dark	78.5[b]	0.91[a]
Photoperiod	85.5[a]	0.61[b]
LSD	3.97	0.08

LSD (0.05) according to the t tests; m1 to m4 are the induction and maintenance media used. For composition of media, please refer to Table 1 Data in columns displaying the same letters are not significantly different at *P = 0.05* according to the t Tests.

FIGURES

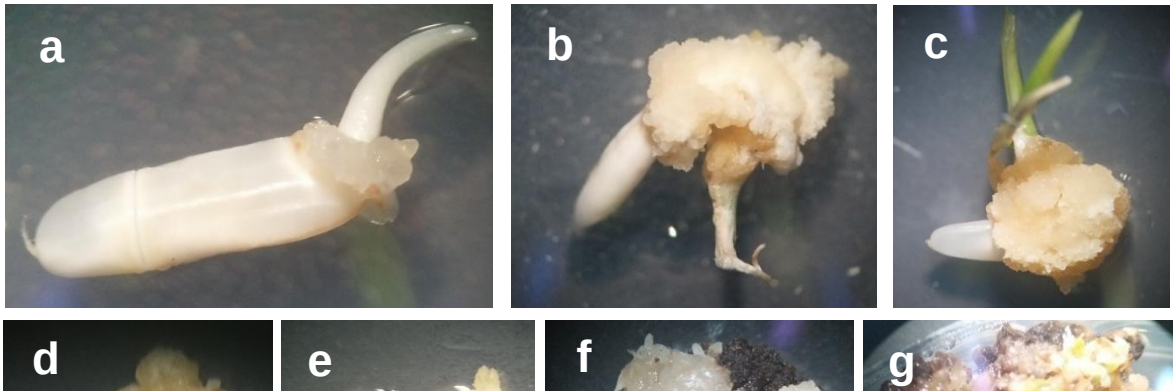

Fig. 1. Callus induction from mature seed of two rice varieties: (a) initiation of callus 4 days after incubation, (b) embryogenic callus 21 days after incubation, (c) organogenic callus 21 days after incubation, (d) embryogenic callus and (e) organogenic callus of 'Nerica 3'obtained at the end of callogenesis after 42 days of culture, (f) embryogenic callus and (g) organogenic callus of 'Nerica L36'obtained at the end of callogenesis after 42days of culture.

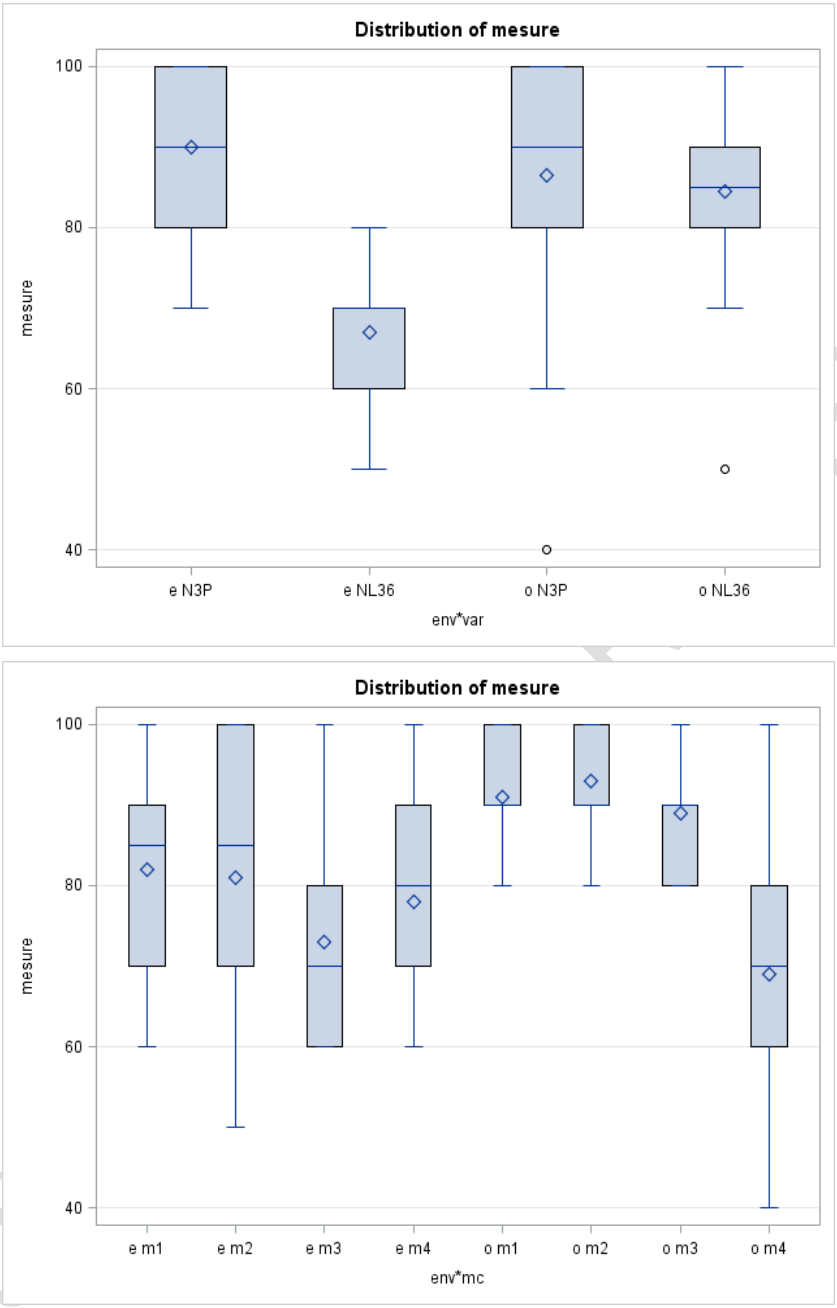

Fig. 2. Effects of variety x environment and medium x environment interactions on callus induction in rice.
e= embryogenic callus obtained in dark environment; o= organogenic callus obtained in photoperiod environment. N3P= "Nerica 3" rice variety; NL36= "Nerica L36" rice variety. m1 to m4 are the induction and maintenance media used. For composition of media, please refer to Table 1.

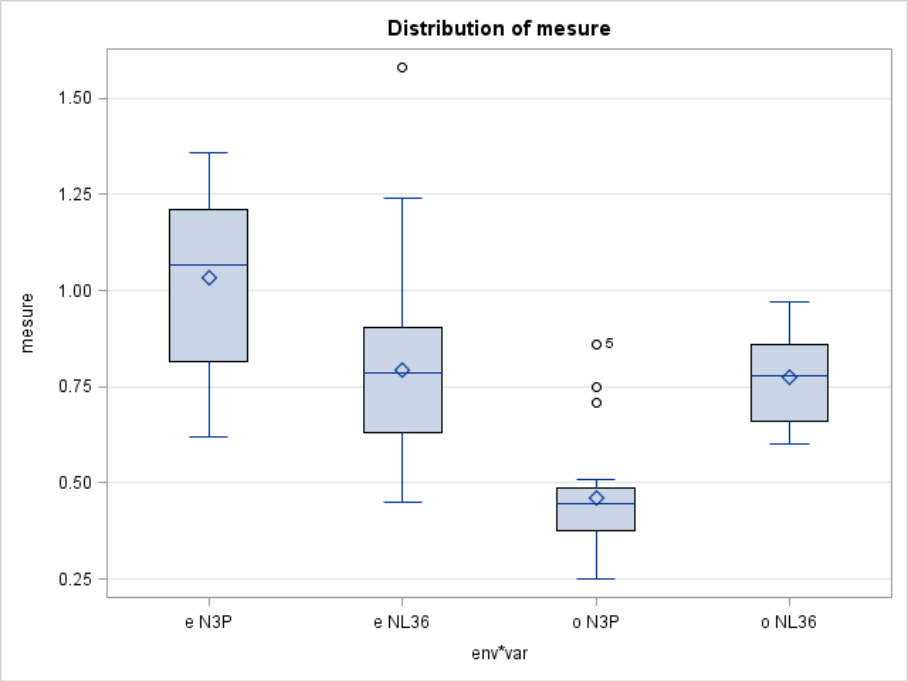

Fig. 3. Effect of variety x environment interaction on callus weight in rice.
e= embryogenic callus obtained in dark environment; o= organogenic callus obtained in photoperiod environment. N3P= "Nerica 3" rice variety; NL36= "Nerica L36" rice variety. m1 to m4 are the induction and maintenance media used. For composition of media, please refer to Table 1.