Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.6, No.18, 2016

Callus Induction and Plant Regeneration from Dehusked Mature Seeds of Three Accessions of African Rice (Oryza glaberrima Steud.)

Rhoda Gyinae Diawuoh [1] George Y. P. Klu [2] Harry M. Amoatey [2] Rosemary Kusi-Adjei [1]
Emmanuel K. Quartey [1]
1. Biotechnology and Nuclear Agriculture Research Institute, Ghana Atomic Energy Commission, P. O. Box LG 80, Legon-Accra, Ghana
2. Department of Nuclear Agriculture and Radiation Processing, Graduate School of Nuclear and Allied Sciences, University of Ghana. P. O. Box AE 1, Atomic Energy- Accra, Ghana

Abstract
Three accessions of African rice *(Oryza glaberrima* Steud.*)*, Guame, N/4 and SARI 1 were assessed for their callus induction and plant regeneration ability from mature dehusked seeds on Murashige and Skoog (MS), (1962) basal medium supplemented with varying concentrations of plant growth regulators. Mature dehusked seeds were inoculated onto callus induction medium consisting of MS supplemented with varying concentrations of 2,4-dichlorophenoxyacetic acid (2,4-D) in the range 0.0-6.0mg/l. All tested accessions exhibited highest callus induction at 4.0 mg/l 2,4-D. Callus induction frequency was significantly ($p \leq 0.05$) different among the accessions, as well as among the 2,4-D levels tested. Calli obtained were also sub-cultured on MS medium, further supplemented with varying concentrations (0.0-2.5 mg/l) of 6-benzylaminopurine (BAP). The highest regeneration frequency was obtained on medium containing 2.0 mg/l BAP. However, callus induced on 3.0 mg/l 2,4-D and further sub-cultured on 2.0 mg/l BAP gave the best response in terms of shoot proliferation, shoot growth and root development; therefore, considered to be the optimum concentrations for callus induction and plant regeneration respectively. Plantlet regeneration was achieved only in the accession N/4 while Guame and SARI 1 exhibited poor regeneration response. Among the three African rice accessions evaluated, N/4 was the most promising in terms of callus induction frequency and regeneration ability.
Keywords: *Oryza glaberrima*, callus induction, plantlet regeneration, shoot proliferation, 2, 4-dichlorophenoxyacetic acid, 6-benzylaminopurine.

1.0 Introduction

African rice *(Oryza glaberrima* Steud) is the indigenous rice species native to, and well adapted to West Africa. It is commonly referred to as red rice because of its red pericap and has many important traits such as weed competitiveness, drought tolerance and ability to respond to low input conditions. It is resistant to pests and diseases, and it is a good source of variation for important abiotic stress factors (Linares, 2002; Nayar, 2010; Van Andel, 2010).

Despite these qualities, *O. glaberrima* species show several negative characteristics such as shattering, brittle grain and poor milling quality compared to the Asian rice species, *O. sativa*. More essentially, it consistently shows lower yields than *O. sativa* (Nayar, 2010).

Conventional breeding has improved tolerance to biotic and abiotic stress factors as well as grain yield in the NERICA varieties,(Jones *et al,* 1997; Sie *et al,* 2005; Samdo *et al* 2008) which have been released to farmers for cultivation in more than 20 countries across Africa (Haskins and Mohapatra, 2010).

However, improvement in grain quality needs urgent attention which can be tackled promptly using direct gene transfer technique, which holds prospects or shortening the breeding cycle (Li *et al.,* 2007).

Agrobacterium mediated transformation of *O. sativa* and other major cereals like maize, wheat and barley has been achieved (Aldemita and Hodges, 1996). However, the improvement of *O. glaberrima* using marker-free transformation biotechnology can be significantly impaired due to lack of a suitable *in vitro* regeneration protocol for the local cultivars. Routine tissue culture system including callus induction and plantlet regeneration are fundamental requirements for successful genetic transformation (Seraj*et al.,* 1997; Li *et al.,* 2007). It is known that callus induction and plant regeneration ability highly depend on genotypes, explant types, culture medium and culture conditions (Rueb*et al.,* 1994). Intraspecific variability, reflected in the genotype-dependent response of *in vitro* cultures, necessitates empirical determination of suitable plant regeneration conditions for individual cultivars (Pierik, 1987; Al-Khayri *et al.,* 1991).

Reliable protocol for callus induction and high frequency plant regeneration are the two prime requirements in rice improvement using this method (Ramesh *et al.,* 2009). Many reports exist on the optimization of tissue culture system of Asian rice cultivars (Zhu *et al.,* 1996; Rashid *et al.,* 2001; Lee *et al.,* 2002; Ge *et al.,* 2006; Zaidi *et al.,* 2006). However, there is no report, so far, on the tissue culture of Ghanaian local rice cultivars. This study seeks to evaluate three Ghanaian *Oryza glaberrima* accessions with respect to

their callus induction and regeneration ability as a potential tool in genetic transformation for improved grain quality of the crop.

2.0 Materials and methods

2.1 Callus induction using dehusked mature seeds of *O. glaberrima.*

Seeds of three accessions of *O. glaberrima* were manually dehusked and surface sterilized with 0.1% mercuric chloride ($HgCl_2$) by vigorously agitating for two minutes under a laminar flow hood and thereafter rinsed with three changes of sterile distilled water. The sterilized seeds were inoculated into honey jars containing 15ml of Murashige and Skoog (MS) (1962) basal medium, supplemented with varying concentrations of 2, 4-D (0.0-6.0 mg/l) together with 30 g/l sucrose, 100 ml/l coconut water, 100 mg/l myo-inositol. The pH of the medium was adjusted to 5.8 using 1 M KOH before the addition of 3.5 g/l phytagel and autoclaving at 121°C for 15 minutes at 15 psi. The cultures were incubated in total darkness at 21°C for eight weeks.

The experiment was set up as a completely randomized factorial design. The factors tested were three accessions of *O. glaberrima* and seven concentrations of 2,4-D. The frequency of callus induction was calculated according to the formula below:

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

Data obtained were subjected to analysis of variance (ANOVA) based on five replications for percentage callus formed. The means were separated, where appropriate, at the 5% significance level using the least significant difference (LSD). Data were analyzed using Genstat statistical package 12th edition.

2.2 Plantlet regeneration from seed-derived calli

Calli obtained from section 2.1 above were sub-cultured on MS medium supplemented with 6-benzylaminopurine (BAP) in the range 0.0-2.5 mg/l, together with 30 g/l sucrose, 100 mg/l myo-inositol. The pH of the medium was adjusted to 5.8 prior to the addition of 3.5 g/1 phytagel. Aliquots of 15ml of the medium were dispensed into 200ml honey jars and autoclaved at 121°C for 15 minutes at 15 psi. The cultures were kept in a growth room at a temperature of 21°C under a 16/8-hr (light/dark) photoperiod with light provided by white fluorescent tubes (T5 fluorescent fitting, UK) at an intensity of 3,000 lux. Three calli clumps were plated per honey jar, with three jars representing a treatment and replicated three times.

The experiment was set up as a completely randomized factorial design. Plantlet regeneration from plated calli was calculated by counting the number of green plants per treatment. Data were subjected to analysis of variance (ANOVA) based on three replications for plantlet regeneration, plantlet height and root formation. The means were separated, where appropriate, at the 5% significance level using the least significant difference (LSD). Data were analysed using Genstat statistical package 12th edition.

2.3 Post-flask acclimatisation of *in vitro* regenerated plantlets

In vitro plantlets regenerated from seed-derived calli of *Oryza glaberrima* were acclimatised in a net house by gradually exposing them to the natural environment. The plantlets were transferred into polyethylene bags filled with soil-cow dung-coconut husk mixture at a ratio of 3:1:1. Plantlets in the polyethylene bags were covered with Watson Modules® to create a humidity chamber thereby reducing transpiration. The set-up was kept in a net house which cuts off 70% of incident sunlight. After three days, the Watson Modules® were removed and the plantlets exposed to the natural environment in the net house. Three weeks after weaning, plantlets were finally transferred to a second net house which cuts off 30% of incident sunlight, for hardening. Survival rate of the plantlets was recorded after six weeks of culture.

3.0 Results and discussion

3.1 Effect of varying concentrations of 2,4-D on callus formation

Mature dehusked rice seeds cultured on MS medium without 2,4-D supplementation germinated, producing seedlings without formation of callus. However, seeds inoculated on MS supplemented with 0.0-2.0 mg/l 2,4-D first exhibited germination, followed by callus formation. Seeds cultured on media containing higher 2,4-D concentrations (3.0-6.0 mg/l), formed callus directly. Soft, friable, creamish calli were observed from the basal region of the germinated seeds two weeks after culture (Figs. 1 C, 1 D). Proliferation of callus continued until the eighth week of culture.

MS medium supplemented with 4.0 mg/l 2,4-D resulted in the highest callus induction of 56.6% while 1.0mg/l 2,4-D resulted in the least (Fig. 2). There was no significant ($p \geq 0.05$) differences among percentage calli formed at 2.0, 3.0, 5.0 and 6.0 mg/l 2,4-D. No callus induction occurred when 2,4-D was excluded from the medium. Earlier researchers have stressed the role of 2,4-D in callus formation in rice tissue culture. Pandey *et al.,* (1994) worked on dehusked rice using different levels of 2,4-D in nutrient medium and concluded that 2,4-D

at a concentration of 2 mg/1 gave the best response for callus formation. Khanna and Raina, (1998) reported that 2,4-D is the most suitable auxin for callus induction of rice in tissue culture, however the optimum concentration of 2,4-D varied depending on the explant source and genotype.

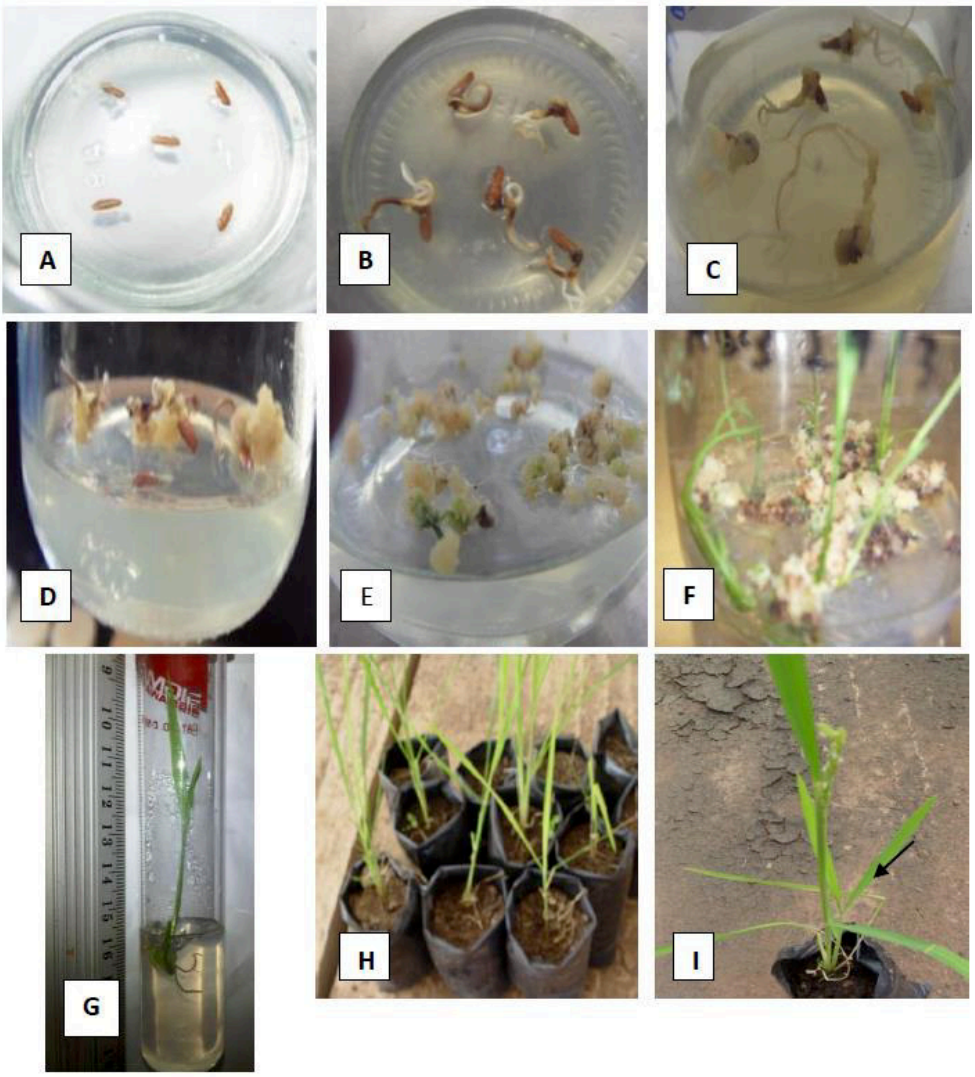

Fig.1 Callus induction and *in vitro* plantlet regeneration from mature seeds of *Oryza glaberrima*.

(A) Dehusked mature seeds inoculated on callus inducing medium
(B) Seeds germinating without callus formation
(C) Seeds initiating callus from coleoptiles
(D) Fully formed callus
(E) Embryogenic calli showing green spots
(F) Regenerated plantlets from calli
(G) *In vitro* regenerated plantlet ready to be weaned
(H) A batch of weaned plants
(I) Regenerated plantlet showing formed panicle.

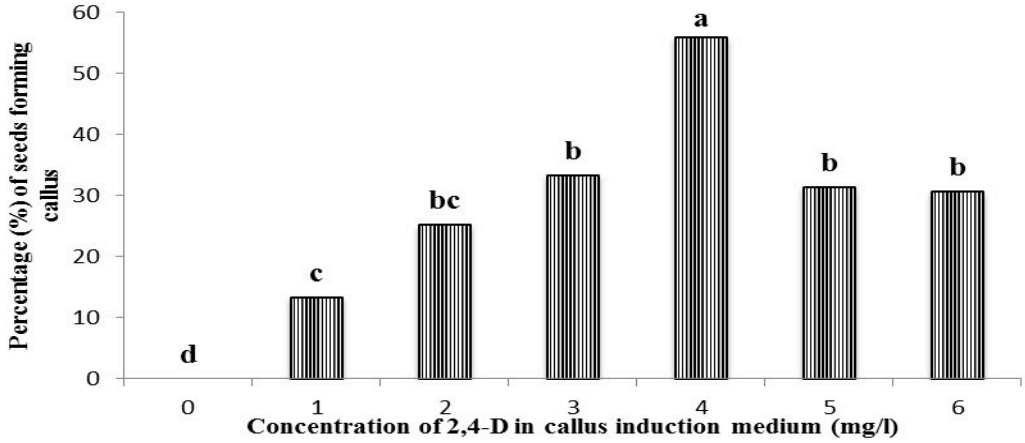

Fig.2 Effect of increasing concentration of 2,4-D in callus induction medium on percentage callus formation (Bars having the same letter are not significantly different (P≥0.05)).

3.2 Response of different *O. glaberrima* accessions to callus induction

Callus induction in the 3 accessions of *O. glaberrima* used for this study was found to be highly variable and genotype specific. Accession N/4 produced 55.4% callus from the inoculated seeds, which was significantly higher than the other 2 accessions (Fig.3). Frequency of callus induction from the seeds of Guame and SARI 1 also differed significantly from each other.

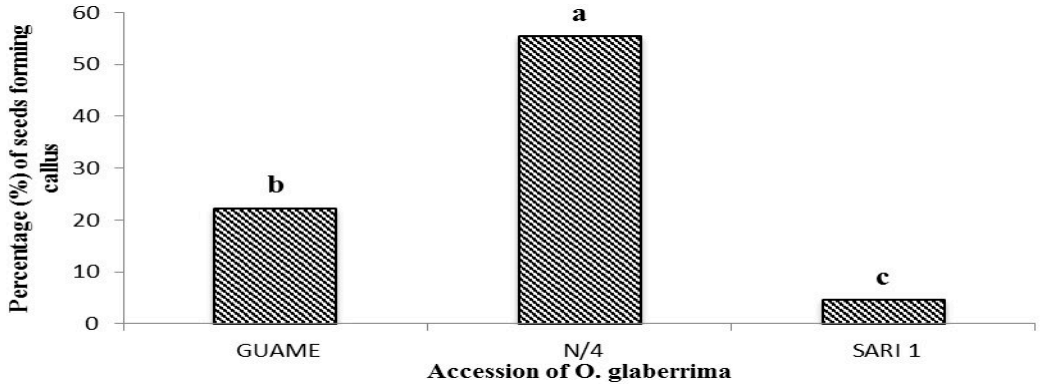

Fig.3 Effect of different accessions of *O. glaberrima* on percentage callus formation (Bars having the same letter are not significantly different (P≥0.05)).

Table 1 displays results of the effect of interaction between accessions and concentration of 2,4-D on callus induction.

Table1: Effect of varying 2,4-D concentration on percentage callus formation in three accessions of *O. glaberrima*

Conc (mg/l) of 2,4-D	Callus formation (%)		
	Guame	N/4	SARI 1
0.0	0.0c	0.0d	0.0
1.0	8.0bc	32.0c	0.0
2.0	16.0bc	60.0b	0.0
3.0	20.0bc	76.0a	4.0
4.0	60.0a	92.0a	16.0
5.0	28.0b	64.0b	8.0
6.0	24.0b	64.0b	4.0

Means in the same column followed by the same letter are not significantly different (P≥0.05).

Callus formation differed significantly among cultivars. In general, as the concentration of 2,4-D increased, callus formation increased at a rate that was accession-specific until an optimum was reached at 4 mg/l. Even though, the highest percentage callus formation was achieved at 4 mg/l 2,4-D for all 3 accessions, the increase in callus formation from 76% on 3 mg/l 2,4-D to 92% on 4 mg/l 2,4-D for N/4 was not statistically significant. However, in Guame the increase from 20% on 3 mg/l 2,4-D to 60% on 4 mg/l 2,4-D was statistically significant. Significant decreases in callus formation occurred in N/4 and Guame when the concentration of 2,4-D was increased beyond 4 mg/l but the same treatment did not cause a significant difference in callus growth for

SARI 1. On media supplemented with 6 mg/1 2,4-D, mean callus formation for the 3 accessions showed no significant differences from the previous concentration.

The study revealed that callus proliferation differed significantly among accessions and also depended upon the concentration of 2, 4-D. Only calli from N/4 grew into plantlets. This observation suggests that N/4 calli were embryogenically competent. This is consistent with observations by Al-Khayri and Anderson, (1995) who in a similar study reported that, the embryogenic competence of induced calli is dependent upon the genotype of the source plant.

3.3 Effect of concentration of 2,4-D in callus induction medium on plantlet development

Table 2 displays results of the effect of concentration of 2,4-D in callus induction medium on number of shoots, shoot height and number of roots per shoot.

Table 2: Effect of varying 2,4-D concentration in callus induction medium on plant regeneration, shoot height and root formation of calli induced from rice seed explants of N/4 accession.

Conc of 2,4-D (mg/l)	Mean number of shoots/culture	Mean number of roots/shoot	Mean height of shoots (cm)
1.0	1.6 ± 0.2^{bc}	4.2± 0.3^{bc}	4.5± 0.3^{b}
2.0	1.9± 0.2^{b}	4.4± 0.3^{b}	6.9± 0.3^{a}
3.0	**4.0± 0.2^{a}**	5.3± 0.3^{a}	**7.4± 0.3^{a}**
4.0	1.6± 0.2^{bc}	**5.8± 0.3^{a}**	3.3± 0.3^{bc}
5.0	1.2± 0.2^{c}	3.5± 0.3^{c}	4.2± 0.3^{b}
6.0	1.1± 0.2^{c}	1.7± 0.3^{d}	2.2± 0.3^{c}

Means in the same column followed by the same letter are not significantly different (P≥0.05).

Upon transfer to regeneration medium, calli from accession N/4 induced on 3.0 mg/l 2,4-D resulted in the highest number of shoots . The highest mean shoot height was also recorded at the same concentration of 2,4-D. However, it was not the same for mean number of roots per shoot for which the highest number was recorded at 4.0 mg/l. A similar trend was observed for mean number of shoots per culture, mean number of roots per shoots and mean height of shoots which values increased with increasing concentration of 2,4-D in the callus induction medium, reaching an optimum and thereafter declining.

The number of shoots produced at 2,4-D concentration of 3.0 mg/l was significantly higher ($p \leq 0.05$) than those produced at other concentrations, all of which were not statistically different. Mean number of roots per shoot increased with increasing 2,4-D concentration in callus induction medium, optimizing at 4.0 mg/l. Thereafter an increase in 2, 4-D concentration caused a decrease in mean number of roots per shoot. Even though, the highest number of roots per shoot was produced at 4.0 mg/l, it was statistically similar to that produced at 3.0 mg/l but differed significantly from those recorded at other concentrations 2,4-D. Generally, from these results shoot proliferation was optimum when callus was induced on MS supplemented with 3.0 mg/l 2,4-D. Shoot elongation and root development peaked at 2.0 to 3.0 mg/l and 3.0 to 4.0 mg/l 2,4-D respectively.

Results of this study revealed an interesting trend showing that the concentration of 2,4-D in callus induction medium can affect the number of plantlets produced by the callus. This is similar to findings by Malik *et al.,* (2004) who in a related study in wheat observed a decreasing trend in shoot formation as concentration of 2,4-D in callus induction medium increased beyond 2.5 mg/l. The reduced number of shoots on higher concentration of 2,4-D could be attributed to the phyto-toxic effect of the higher concentration of 2,4-D, suppressing the initiation of shoots from callus. Indeed, the phyto-toxic effect of the high concentration of 2,4-D have long been established and exploited commercially in the production of herbicedes.

3.4 Effect of concentration of BAP in regeneration medium on plantlet development

Table 3 displays results of the effect of concentration of BAP in regeneration medium on number of shoots per culture, shoot height and number of roots per shoot.

When calli were transferred to regeneration media, some calli exhibited no response at all or became necrotic, while others developed into either roots only or shoots with roots (full plantlets) depending on the accession. Plantlet regeneration was observed as early as 2 weeks after calli were placed on regeneration medium, especially from calli that had formed on medium supplemented with low concentrations of 2,4-D (1.0 - 3.0) mg/l. The cultures were maintained for 12 weeks to allow time for potential regenerative calli to respond. Observations made at the end of the culture period revealed that the percentages of embryogenic and rhizogenic calli were variable among genotypes. Only calli induced from N/4 accession were found to be embryogenic.

Table 3: Effect of varying BAP concentration in regeneration medium on plantlet regeneration, shoot height and root formation of calli induced from rice seed explants of accession N/4.

Conc of BAP (mg/l)	Mean number of shoots/culture	Mean number of roots/shoot	Mean height of shoots (cm)
0.0	0.1± 0.2^{c}	0.7± 0.3^{c}	0.9± 0.3^{c}
0.5	1.9± 0.2^{b}	3.8± 0.3^{b}	4.4± 0.3^{b}
1.0	2.0± 0.2^{b}	5.1± 0.3^{a}	5.7± 0.3^{ab}
1.5	2.1± 0.2^{b}	5.2± 0.3^{a}	6.4± 0.3^{a}
2.0	**3.0± 0.2^{a}**	**5.4± 0.3^{a}**	**6.5± 0.3^{a}**
2.5	2.2± 0.2^{b}	4.7± 0.3^{a}	4.6± 0.3^{b}

Means in the same column followed by the same letter are not significantly different (P≥0.05).

Mean number of shoots per culture increased with increasing concentration of BAP in regeneration medium from 0.0 - 2.0 mg/l. Thereafter, mean number of shoots decreased following an increase in BAP concentration from 2.0 mg/l to 2.5 mg/l. Number of shoots obtained from medium supplemented with 2.0 mg/l BAP was significantly higher than those obtained from the other concentrations. There were no significant differences in number of shoots produced at BAP concentrations of 0.5, 1.0, 1.5 and 2.5 mg/l.

Mean shoot height also increased with increasing concentration of BAP in culture medium. Shoot elongation was at its peak when callus was sub-cultured on medium containing 2.0 mg/l BAP. Mean shoot height decreased significantly when concentration of BAP was increased further beyond 2.0 mg/l. However, mean shoot height recorded at concentration of 2.0 mg/l BAP was statistically similar to that recorded at concentrations of 1.0 and 1.5 mg/l BAP.

Similarly, mean number of roots per shoot increased as concentration of BAP in regeneration medium increased reaching an optimum at 2.0 mg/l. Mean number of roots however, declined with further increase in concentration of BAP beyond 2.0 mg/l. Even though, the highest number of roots per shoot was produced at concentration of 2.0 mg/l BAP, it did not differ significantly from those produced at concentrations of 1.0, 1.5 and 2.5 mg/l BAP.

From the results, it is evident that shoot proliferation, shoot elongation and root development were at their best performance when callus was sub-cultured on MS supplemented with 2.0 mg/l BAP as the highest number of plantlets, highest number of roots as well as the tallest shoots were produced at this concentration.

The presence of BAP in regeneration medium played an important role of stimulating the formation of shoots. Concentration of BAP in the regeneration medium was directly proportional to the number of shoots recovered until an optimum was reached at 2.0 mg/l after which an increase in BAP level did not yield a corresponding increase in shoot numbers. The results indicate that regeneration of shoots is influenced by both the presence and concentration of BAP in the regeneration medium and are in agreement with reports by Shaheenuzzaman *et al*., (2011) and Malek *et al.,* (2007), who in similar studies in *Gladiolus spp* and *Trichosanthes dioica* respectively, observed that the absence of BAP in regeneration media gave low numbers of shoots but these increased when concentration was increased. They also reported that an increase beyond the optimum levels, which were 3.0 mg/l and 2.0 mg/l for *Gladiolus* and *Trichosanthes* respectively, led to reduced shoot numbers. This implies that higher concentrations adversely affected the rate of shoot formation. The adverse effect of higher concentrations of cytokinins on shoot development has been reported in many plant species (Thoyajasksha and Ravishankar, 2001; Nasib *et al*., 2008; Kalidass and Mohan, 2009). This adverse effect of cytokinins on shoot proliferation may be due to phytotoxicity resulting from an excess concentration of the growth regulator in the explant as a result of addition of the same growth regulator from exogenous source (Werner *et al.,* 2001).

3.5 Effect of interaction of 2,4-D and BAP on plantlet development

Effect of interaction between 2,4,-D and BAP on mean number of shoots per cultures, height of shoots and mean number of roots per shoot are displayed in Table 4.

Table 4: Effect of interaction of 2,4-D and BAP concentrations on plantlet regeneration, shoot height and root formation of calli induced from rice seed explants of N/4 accession.

Conc of 2,4-D (mg/l)	Conc of BAP (mg/l)	Mean number of shoots/culture	Mean height of shoots (cm)	Mean number of roots/shoot
1.0	0.0	0.3± 0.8^{hi}	1.7± 2.0^{ij}	1.0± 1.2^{mn}
	0.5	1.0± 0.8^{g}	4.7± 2.0^{defghi}	4.3± 1.2^{fghijkl}
	1.0	2.3± 0.8^{def}	4.6± 2.0^{defghi}	4.7± 1.2^{ghijk}
	1.5	2.0± 0.8^{efg}	5.4± 2.0^{defgh}	5.3± 1.2^{efgh}
	2.0	2.3± 0.8^{def}	5.5± 2.0^{defgh}	5.5± 1.2^{defgh}
	2.5	1.7± 0.8^{fg}	5.2± 2.0^{defgh}	4.5± 1.2^{fghijkl}
2.0	0.0	0.0± 0.8^{i}	0.0± 2.0^{j}	0.0± 1.2^{n}
	0.5	2.7± 0.8^{def}	6.4± 2.0^{cdef}	2.7± 1.2^{lm}
	1.0	2.0± 0.8^{efg}	**15.3**± 2.0^{a}	7.3± 1.2^{bcd}
	1.5	1.7± 0.8^{fg}	7.6± 2.0^{cd}	5.2± 1.2^{efgh}
	2.0	3.3± 0.8^{cd}	6.6± 2.0^{cdef}	4.5± 1.2^{fghijkl}
	2.5	1.7± 0.8^{fg}	5.4± 2.0^{defgh}	6.8± 1.2^{bcde}
3.0	0.0	0.3± 0.8^{hi}	3.6±2.0^{fghi}	3.2± 1.2^{ijkl}
	0.5	3.0± 0.8^{de}	5.1± 2.0^{defgh}	5.1± 1.2^{efghi}
	1.0	4.3± 0.8^{bc}	9.5±2.0^{bc}	5.0± 1.2^{efghij}
	1.5	4.7± 0.8^{b}	7.0± 2.0^{cde}	4.6± 1.2^{fghijkl}
	2.0	**6.3± 0.8**^{a}	12.0±2.0^{b}	7.7± 1.2^{abc}
	2.5	5.3± 0.8^{ab}	6.9± 2.0^{cde}	6.0± 1.2^{cdef}
4.0	0.0	0.0± 0.8^{i}	0.0± 2.0^{j}	0.0± 1.2^{n}
	0.5	3.0± 0.8^{d}	4.7± 2.0^{defghi}	5.9± 1.2^{cdefg}
	1.0	2.3± 0.8^{def}	4.3± 2.0^{efghi}	**9.3± 1.2**^{a}
	1.5	1.3± 0.8^{fgh}	3.3± 2.0^{ghi}	8.5± 1.2^{ab}
	2.0	1.7± 0.8^{fg}	4.0± 2.0^{efghi}	6.8± 1.2^{bcde}
	2.5	1.3± 0.8^{fgh}	3.3± 2.0^{ghi}	4.2± 1.2^{fghijkl}
5.0	0.0	0.0± 0.8^{i}	0.0± 2.0^{j}	0.0± 1.2^{n}
	0.5	2.0± 0.8^{efg}	5.3± 2.0^{defgh}	4.7± 1.2^{fghijk}
	1.0	1.3± 0.8^{fgh}	4.4± 2.0^{efghi}	4.0± 1.2^{ghijkl}
	1.5	1.0± 0.8^{g}	6.9± 2.0^{cde}	4.6± 1.2^{fghijkl}
	2.0	1.7± 0.8^{fg}	4.8± 2.0^{defghi}	3.8± 1.2^{hijkl}
	2.5	1.0± 0.8^{g}	3.6± 2.0^{fghi}	3.9± 1.2^{hijkl}
6.0	0.0	0.0± 0.8^{i}	0.0± 2.0^{j}	0.0± 1.2^{n}
	0.5	0.0± 0.8^{i}	0.0± 2.0^{j}	0.0± 1.2^{n}
	1.0	0.0± 0.8^{i}	0.0± 2.0^{j}	0.0± 1.2^{n}
	1.5	1.3± 0.8^{fgh}	3.7± 2.0^{fghi}	3.0± 1.2^{kl}
	2.0	2.7± 0.8^{def}	6.3± 2.0^{cdef}	4.1± 1.2^{fghijkl}
	2.5	2.3± 0.8^{def}	3.2± 2.0^{hi}	3.1± 1.2^{jkl}

Means in the same column followed by the same letter are not significantly different (P≥0.05).

2,4-D at a concentration of 3.0 mg/l followed by BAP at 2.0 mg/l was most effective, as it produced the highest (p≤0.05) number of shoots per culture. Maximum shoot height was attained at a combined hormone concentration of 2.0 mg/l 2,4-D and 1.0 mg/l BAP. Highest number of roots per shoot were observed in callus generated on medium containing 2,4-D concentration of 4.0 mg/l and sub- cultured on medium fortified with BAP at a concentration of 1.0 mg/l.

In general, the highest number of shoots per culture, number of roots per shoot and the tallest plantlets were achieved when callus was induced on 3.0 mg/l, 2.0 mg/l and 4.0 mg/l 2,4-D respectively and sub-cultured on MS supplemented with concentrations of 2.0 mg/l, 1.0 mg/l and 1.0 mg/l BAP respectively. On the other hand, the least number of shoots per culture, least numbers of roots and the shortest plantlets were recorded when the regeneration medium was devoid of BAP irrespective of the concentration of 2,4-D that yielded the calli.

Results from interaction of 2,4-D and BAP show that the combined effect of concentration of 2,4-D from which callus was induced and concentration of BAP on which callus was sub-cultured leading to the regeneration of plantlets played a significant role in determining the number of shoots recovered and subsequently the shoot height and number of roots per shoot. Earlier workers (Malek *et al.,* 2007; Shaheenuzzaman *et al.,* 2011) reported that callus induced on lower concentration of auxin has much potential and produces more plantlets per callus when sub-cultured on medium containing fairly high levels of cytokinin. The current findings are in agreement with results obtained by these earlier workers in that, callus induced on

low levels of 2,4-D (1.0 - 3.0 mg/l) regenerated plantlets in slightly higher numbers when transferred to medium with BAP concentration of 2 mg/l.

Post flask acclimatisation

Ninety percent (90%) of regenerated plantlets survived in soil irrespective of *in vitro* treatments. Under net house conditions, the regenerants exhibited normal growth and produced viable seeds. However, *in vitro* regenerated plants took 32 days to boot and produced fewer tillers per stand (1-3) while the parent material booted in 45 days producing higher number (3-7) of tillers per stand. This may be due to induction of somaclonal variation.

Conclusion

The optimum concentration of 2,4-D for callus induction using dehusked mature seed ex-plants of *O. glaberrima* is 4.0 mg/l. Similarly, the optimum concentration of BAP for plantlet regeneration for seed-derived callus of *O. glaberrima* is 2.0 mg/l. Maximum shoot proliferation, root development and plantlet growth were observed when callus was induced on 3.0 mg/l 2,4-D and sub-cultured on 2 mg/l BAP. Of the three accessions evaluated only N/4 showed embryogenic competence and therefore is recommended for further studies.

REFERENCES

Aldemita, R. R, Hodges, T. K, (1996), *Agrobacterium tumefaciens*-mediated transformation of *japonica* and *indica* rice varieties, *Planta*, 199, 612-617

Al-Khayri, J. M, Huang, F. H, Morelock, T. E, Busharar, T. A, Gbur, E. E, (1991), Genotype dependent response of spinach cultivars to *in vitro* callus induction and plant regeneration, *Plant Sci.,* 78, 121-127.

Ge, .X. J, Chu, Z. H, Lin, Y. J, Wang, S. P, (2006), A tissue culture system for different germplasms of *indica* rice, *Plant Cell Rep*., 25, 392-402.

Haskins, J, and Mohapatra, S, (2010), Endangered African Rice Varieties gain elite status, *Eurek Alert Public Release*, pp 3

Jones, M. P, Dingkuhn, M, Aluko, G. K, Semon, M, (1997), Interspecific *Oryza sativa* L x *Oryza glaberrima* Steud. Progenies in upland rice improvement, *Euphytica*, 94: 237-246.

Kalidass, C, Mohan, V. R, (2006), *In vitro* rapid clonal propagation of *Phyllanthus urinaria* L. (Euphorbiaceae): A medicinal plant. *Researcher,* 1(4): 56-61.

Khanna, H. K, Raina S. K, (1998), Genotype x culture media interaction effects on regeneration response of 3 indica rice cultivar, *Plant Cell, Tissue Organ Cult.*, 52, 145-153.

Lee, K. S, Jeon, H. S, Kim, M. Y, (2002), Optimization of a mature embryo based *in vitro* culture system for high-frequency somatic embryogenic callus induction and plant regeneration from japonica rice cultivars, *Plant Cell Tissue Organ Cult.,* 71, 9-13.

Li L, Duan S, Kong J, Li S, Li, Y, Zhu Y, (2007), A single genetic locus in chromosome 1 controls conditional browning during the induction of calli from mature seeds of *Oryza sativa* ssp. *Indica, Plant Cell Tissue Organ Cult.,* 89, 237-245.

Linares, O. F. (2002), African rice (*Oryza glaberrima)* History and future potential, PNAS 99 (25) 16360-16365.

Malek, M. A, Miah, M. A. B, Al-Amin, M, Khanam, D, Khatun, M, (2007), I*n vitro* regeneration in pointed gourd, *Bangladesh J. Agril. Res.,* 32(3), 461-471

Malik, S. I, Rashid, H, Yasmin, T, Minhas, M. N, (2004), Effect of 2,4-dichlorophenoxyacetic acid on callus induction from mature wheat (*Triticum aestivum* L.) seeds, *Int. J. Agric. Biol.,* 6(1), 156-159

Murashige T, Skoog F, (1962), A revised medium for rapid growth and bioassays with tobacco tissue culture, *Physiol. Plant.,* 15 (3), 473-497

Nasib, A, Ali, K, Khan, S, (2008), *In vitro* propagation of Croton (*Codiaeum variegatum*), *Pak. J. Bot.,* 40(1), 99-104.

Nayar, N. M, (2001), The history and genetic transformation of the African rice, *Oryza glaberrima* Steud, *Current Sci.*, 99(12), 1681-1689

Pandey, S. K, Ramesh, B, Gupta, P. K. S, (1994), Callusing and plant regeneration in rice, *Indian J. Genet.,* 54(3), 293- 299.

Pierik, R. L. M, (1987), *In vitro culture of higher plants,* Martinus Nijhoss Publishers, Dordrecht, The Netherlands, pp 344.

Ramesh, M, Murugiah, V, Gupta, K. A, (2009), Efficient *in vitro* plant regeneration via leaf base segments of *indica* rice (*Oryza sativa* L.), *Indian J. Exp. Biol.,* 47, 68-74

Rashid, H, Bokhari, S. Y. A, Quraishi A, (2001), Callus induction, regeneration and hygromycin selection of rice (Super Basmati*), Online J. Biol. Sci.,* 1, 1145-1146.

Rueb, S, Leneman, M, Schilperoort, R. A, Hensgens, L. A. M, (1994), Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa* L.), *Plant Cell Tissue Organ Cult.,* 36, 259-264.

Seraj, Z. I, Islam, Z, Faruque, M. O, Devi T, Ahmed S, (1997), Identification of the regeneration potential of embryo derived calluses from various indica rice varieties, *Plant Cell Tissue Organ Cult.,* 48, 9-13.

Shaheenuzzaman, M, Haque, M. S, Karim, M. M, Noor, Z. U, (2001), *In vitro* shoot proliferation and development of micropropagation protocol from leaf disc of Gladiolus, *J. Bangladesh Agril. Univ.,* 9(1), 21–26.

Sie, M, Dogbe, S. Y, Coulibaly, M, (2005), Selection of interspecific hybrids (*O. sativa x O. glaberrima* or lowland NERICAs) and intraspecifics adapted to rainfed low growing condition. *Int. Rice Comm. Newslett*, 54:47-51

Somado, E. A, Guei, R. G, Keya, S. O, (2008), NERICA: The New Rice for Africa: A Compendium. Africa Rice Centre (WARDA), Cotonou, Benin, ISBN-13: 9789291133178, page 195.

Thoyajasksha, S, Ravishankar, R, (2001), *In vitro* micropropagation of *Dictyospermum ovalifolium* Wight. A rare endemic plant in Western Ghats India, *Plant Cell Biol. & Molec. Biol.,* 2, 57-62.

Van Andel T, (2010), African rice (*Oryza glaberrima* Stued.): Lost crop of the enslaved Aficans, discovered in Suriname, Econ Bot 64 (1) 1-10.

Werner, T., Motyk, V., Strnad, M., Schmullihg, T. (2001), Regulation of plant growth by cytokinin, PNAS, 98 (18) 10487-10492.

Zaidi, M. A, Narayanan, M, Sardana, R, Taga, I, Postel, S, Johns, R, McNulty, M, Mottiar, Y, Mao, J, Loit, E, Altosaar, I, (2006), Optimizing tissue culture media for efficient transformation of different indica rice genotypes, *Agron. Res.,* 4, 563-575.

Zhu, X. Y, Ouyang, W. J, Li, Y, Chen, Z. L, (1996), The effects of 2iP and 2,4-D on rice calli differentiation, *Plant Growth Regul.,* 19, 19-24.