COBISS Code 1.01

DOI: 10.2478/acas-2013-0019
Agrovoc descriptors: oryza sativa, rice, regeneration, callus, callogenesis, stems, plant anatomy, embryonic development, biological development

Agris category code: f50, f62

Indirect plant regeneration in aromatic rice (*Oryza sativa* L.) var. 'Kalijira' and 'Chinigura'

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Received May 11, 2013; accepted August 27, 2013.
Delo je prispelo 11. maja 2013, sprejeto 27. avgusta 2013.

ABSTRACT

Mature seeds of two traditional rice genotypes (Kalijira and Chinigura) were used for callus induction and plant regeneration on different concentrations and combinations of plant growth regulators cultured on MS (Murashige and Skoog) basal medium. Callus induction frequency was different between the cultivars, as well as among the 2,4-dichlorophenoxyacetic acid (2,4-D) levels tested. Both tested cultivars exhibited highest callus frequency at 2 mgl^{-1} 2,4-D. The incorporation of benzylaminopurine (BAP) and kinetin (KIN) in the callus induction medium supplemented with 2 mgl^{-1} 2,4-D did not significantly improve the callus induction frequency but required days of callus initiation were decreased compared to single use of 2,4-D. After two subcultures, at 21 days interval, embryogenic callus was placed on medium containing different concentration and combination of auxin and cytokinin. Treatment T_4 (0.5 mg l^{-1} BAP and 0.1 mg l^{-1} IBA) showed the highest shoot induction: 91.67% in Kalijira and 83.33% in Chinigura. Similarly, the highest range of shoot number was also observed in both genotypes when treated with 0.5 mgl^{-1} BAP and 0.1 mgl^{-1} IBA. Plant regeneration efficiency was further observed best when treated with 1 mgl^{-1} 2,4-D along with 1 mgl^{-1} 2,4-D along with 1 mgl^{-1} BAP and 1 mgl^{-1} IBA. Furthermore, the highest number of callus derived shoot per culture was achieved in 2 mgl^{-1} 2,4-D along with 1 mgl^{-1} BAP and 1 mgl^{-1} IBA. Both rice genotypes are promising in terms of callus induction frequency and morphology, and regeneration ability of the embryogenic callus.

Key words: callus induction, plant regeneration, aromatic rice, shoots

IZVLEČEK

POSREDNA REGENERACIJA AROMATIČNEGA RIŽA (*Oryza sativa* L.), SORT 'KALIJIRA' IN 'CHINIGURA'

Zrela semena dveh tradicionalnih genotipov riža ('Kalijira' and 'Chinigura') so bila uporabljena za indukcijo kalusa in regeneracijo rastlin pri različnih koncentracijah in kombinacijah rastlinskih rastnih regulatorjev pri gojenju na osnovnem MS (Murashige and Skoog) mediju. Frekvenca indukcije kalusa je bila različna med sortama kot tudi glede na koncentracije 2,4-diklorfenoksi ocetne kisline (2,4-D). Obe preiskušeni sorti sta imeli največjo frekvenco kalusa pri 2 mgl^{-1} 2,4-D. Dodatek benzilaminopurina (BAP) in kinetina (KIN) v medij za indukcijo kalusa z dodatkom 2 mgl^{-1} 2,4-D ni značilno izboljšal indukcije kalusa, vendar so se potrebni dnevi za začetek tvorbe kalusa zmanjšali v primerjavi s postopkom, ko smo uporabili samo 2,4-D. Po dveh predkulturah, v interval 21 dni, je bil embriogeni kalus prenešen na medij, ki je vseboval različno koncentracijo in kombinacijo auksina in citokinina. Tretma T_4 (0.5 mg l^{-1} BAP in 0.1 mg l^{-1} IBA) je dal največjo indukcijo poganjkov: 91.67 % pri 'Kalijira' in 83.33 % pri 'Chinigura'. Podobno je nastalo največ poganjkov pri obeh sortah, kadar so jih tretirali z 0.5 mgl^{-1} BAP in 0.1 mgl^{-1} IBA. Nadalje je bila sposobnost regeneracije rastlin najboljša, če so jih tretirali z 1 mgl^{-1} 2,4-D z dodatkom 1 mgl^{-1} BAP in 1 mgl^{-1} IBA. Največje število iz kalusa nastalih poganjkov na kulturo je bilo doseženo pri 2 mgl^{-1} 2,4-D z dodatkom 1 mgl^{-1} BAP in 1 mgl^{-1} IBA. Oba genotipa riža sta obetavna v smislu morfologije in pogostosti indukcije kalusa kot tudi v regeneracijski sposobnosti embriogenega kalusa.

Ključne besede: indukcija kalusa, regeneracija rastlin, aromatični riž, poganjki

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1 INTRODUCTION

Global population is increasing very rapidly. Loss in crop production could lead to hunger and famine, especially in the developing countries. So it is time to tackle the challenges of a rapidly increasing population and stiffer global competition in the next millennium. Moreover these two challenges require better research to produce more and better quality food efficiently. The improvement can possibly be achieved by creating genetic variability. Rice (*Oryza sativa* L.) is the world most important food supplier cereal crop after wheat and maize (Ray, 1985). It provides half of total dietary carbohydrate, especially in Asian countries and it is suitable diet for more than three billion people, supplying 50-80% of their daily calorie intake (Khush, 2005). Thus a considerable improvement has been done through traditional rice breeding. Rice breeding has made significant progress towards higher yield, improved quality, greater disease resistance and other important characters of agricultural importance in the past and even in future, it will still play an important role. Due to its increasing importance in nutrition and economy, it is now felt that new varieties of rice, having good agronomic characters, should be evolved.

Kalijira and Chinigura are the most important aromatic rice varieties of Bangladesh and the rest of the world due to its attractive flavor, fine grain and good taste. Aroma and taste are caused by the chemical compound 2-acetyl-1-pyrroline (Ghareyazie *et al*., 1997). This rice is generally used to prepare dishes such as *polau, biriani* and different types of cake which are served on special occasions. Aromatic rice receives premium price and is profitable for the growers as well as the traders. Country can benefit by earning exchange by production and export of aromatic rice.

Most of the aromatic rice cultivars are traditional rice varieties which have tall stature, low yield, photoperiod-sensitivity, are susceptible to disease and pest and unresponsive to fertilizer. But due to the favorite flavor and some other dominant grain quality characteristics, they are the important resource for breeding and improving the aromatic rice cultivars for diverse demands of consumers in the world. Several laboratories have described regeneration of plants from various rice explants such as immature embryos, immature panicles (Ling *et al*., 1983), young inflorescence (Chen *et al*., 1985) and root (Abe and Futsuhara, 1985). Rashid *et al*. (2000) studied that rice seeds have more potential for callogenesis as compared to node or tip. Successful callus induction from rice seed has been reported by several researchers (Gonalz, 2000; Alam *et al*., 2003; Shahsavari *et al*., 2010). The use of mature seeds has the advantage, because they can be obtained at anytime throughout the year regardless of growing season (Alam, 1994). Despite the enormous importance of aromatics rice, knowledge on the *in vitro* propagation of these rice lines is still elusive.

Therefore, this study was aimed at evaluating two Bangladeshi aromatic rice genotypes (Kalijira and Chinigura) for callus induction and regeneration efficiency under different concentrations and combinations of growth regulators.

2 MATERIALS AND METHODS

2.1 Explant sterilization and culture establishment

Mature seeds of two genotypes of aromatic rice namely; Kalijira and Chinigura were dehusked and immersed in 70% ethanol for 3 min, after washing the explants were dipped in 0.1% $HgCl_2$ solution for 3 minutes. The seeds were then rinsed 5-6 times with sterile distilled water to remove $HgCl_2$ with vigorous agitation in the laminar air flow cabinet. After surface sterilization of seeds, they were kept on autoclaved filter paper on the petridish. When the water removed from the seeds surface it was inoculated into the culture tubes with sterilized forceps. The seeds were then placed on callus induction media and kept in the dark at 26 ± 2°C. MS (Murashige and Skoog, 1962) basal medium was used for callus induction and plant regeneration. In this study, 30 mgl^{-1} sugars was used and solidified with 0.8% agar. The pH of the medium was adjusted to 5.8.

2.2 Callus induction

Different concentrations of 2,4-D (1, 2, 3 and 4 mgl^{-1}) were added into the MS medium for callus induction. Subculture was performed twice at 21-day interval using the same medium. Combinations of auxin and cytokinin (T_1=2.0 mgl^{-1} 2,4-D+0.25 mgl^{-1} KIN, T_2=2.0 mgl^{-1} 2,4-D+0.5 mgl^{-1} KIN, T_3=2.0 mgl^{-1} 2,4-D+1.0 mgl^{-1} KIN, T_4=2.0 mgl^{-1} 2,4-D+1.5 mgl^{-1} KIN, T_5=2.0 mgl^{-1} 2,4-D+0.25 mgl^{-1} BAP, T_6=2.0 mgl^{-1} 2,4-D+0.5 mgl^{-1} BAP, T_7=2.0 mgl^{-1} 2,4-D+1.0 mgl^{-1} BAP, T_8=2.0 mgl^{-1} 2,4-D+1.5 mgl^{-1} BAP mgl^{-1}) were also used in MS media for callus induction.

2.3 Plant regeneration

Embryogenic calli produced on MS medium containing 2 mgl^{-1} 2,4-D were cultured on different regeneration media for plantlet formation. MS basal media supplemented with different concentrations and combinations of cytokinin and auxins (T_1=0, T_2=0.1 mgl^{-1} IBA, T_3=0.1 mgl^{-1} BAP+0.1 mgl^{-1} IBA, T_4=0.5 mgl^{-1} BAP+0.1 mgl^{-1} IBA, T_5=1 mgl^{-1} BAP+0.5 mgl^{-1} IBA, T_6=0.5 mgl^{-1} BAP+0.1 mgl^{-1} IAA, T_7=0.5 mgl^{-1} BAP+0.5 mgl^{-1} IBA, T_8=3 mgl^{-1} KIN+0.5 mgl^{-1} NAA, T_9=3 mgl^{-1} KIN+0.5 mgl^{-1} IAA mgl^{-1}) were prepared for plantlet regeneration. Regenerated shoots were then transferred to half MS media immediately under light (2000 lux) provided by 40W white cool fluorescence tubes. The cultures were maintained in a growth chamber at 24 + 18^0C for a 16 h photoperiod under cool white fluorescent lamps (Phillips Bangladesh Ltd.) and the light intensity was maintained at 28–34 mol/m/s.Visual observation of culture was made every week.

2.4 Data recording

The frequency of callus induction and plant regeneration (%) were measured using the following formulas (Zaidi *et al*., 2006):

$$\text{Frequency of callus induction } (\%) = \frac{\text{no. of explants induced callus}}{\text{no. of cultured explants}} \times 100$$

$$\text{Frequency of shoot induction } (\%) = \frac{\text{no. of culture induced shoot}}{\text{no. of culture}} \times 100$$

$$\text{Frequency of root induction } (\%) = \frac{\text{no. of shoot induced root}}{\text{no. of culture inducted shoot}} \times 100$$

2.5 Statistical analysis

The experiments were arranged in a split plot design with three replications. Each replication per treatment contained 12 seeds for callus induction and 4-6 embryogenic calli for plant regeneration. Data were analyzed using the two way-factorial analysis of variance (factorial ANOVA), with plant growth regulator concentration as one treatment and genotype as the other treatment. Data were analyzed as means ± SE. IRRIState 7.2 software was also used to do ANOVA and DMRT.

3 RESULTS

3.1 Effect of 2,4-D on callus induction

Different concentrations (1.0, 2.0, 3.0 and 4.0 mgl^{-1}) of 2,4-D were used for producing sufficient amount of embryonic callus from mature seeds in MS medium. The results are presented in Table 1. Results indicate that growth regulators played a major role in callus induction. The callus induction was occurred at 7-12th days after inoculation.

Result showed that MS medium supplemented with 2 mgl^{-1} of 2,4-D was most effective in callus induction in both Kalijira (97.22%) and Chinigura (94.44%). This indicates that the use of 2,4-D with 2 mgl^{-1} was enough for production of high amount of callus in rice. Lowest range of days for callus induction was observed in both Kalijira and Chinigura in higher (4.0 mgl^{-1}) concentration of 2,4-D. The color of all Kalijira callus was creamy yellowish and Chinigura was creamy white but both of those textures were friable.

Table 1: Effect of 2,4-D in MS media on quality and quantity of callus induction.

	Kalijira			Chinigura			
Concentration of 2,4-D mgl^{-1}	**Range**	**%**	**Degree with callus morphology**	**Range**	**%**	**Degree with callus morphology**	**Mean (varieties)**
1	10-12	91.66±1.3 a	+++Py,C	13-15	86.11±2.1 a	+++CrW,C	88.89±2.7
2	7-10	97.22±0.8 a	+++Py,C	11-13	94.44±0.8 a	+++CrW,C	95.83±1.4
3	7-10	94.44±0.8 a	+++Py,C	11-13	91.66±1.4 a	+++CrW,C	93.21±1.4
4	8-11	88.79±0.8 a	+++Py,C	12-15	88.78±0.8 a	++CrW,C	88.78±0.0
Mean (treatments)		93.0±1.7			90.2±1.7		

+ Slight callus, ++ Moderate callus, +++ Massive callus, Py= Pale yellow, C=Creamy and CrW= Creamy white; concentrations with the same letter were not significantly different at 0.05 probability level using LSD.

3.2 Effect of 2,4-D in combination with KIN and BAP on induce callus

Although 2,4-D (auxin) gave the highest result of callus induction in rice, some worker have showed a good result in other cereal crops (e.g. wheat) using 2,4-D in combination with low concentration of cytokinine.

The effect of cytokinin (BAP and KIN) along with (2,4-D, 2.0 mgl^{-1}) on callus induction was also tested in MS medium (result shown in Table 2). KIN was found more effective (95.8±1.4) than BAP (94.4±0.0) for high amount of callus formation. In addition, numerous callus (95.8±1.4) was observed when explants were treated with 2.0 mgl^{-1} of 2,4-D was supplemented with 0.5 mgl^{-1} of KIN . Similar result was also found when treated with 2.0 mgl-1 of 2,4-D (95.83 %). But required days of callus initiation were decreased (5-7 days) by using cytokinins along with 2,4-D than single use of 2,4-D in all cases.

Table 2: Effect of different combinations of growth regulator on callus initiation and callus growth.

	Kalijira			Chinigura			
Concentrations and combinations	**Range**	**%**	**Degree**	**Range**	**%**	**Degree**	**mean**
T_1	6-7	94.44±0.8 a	+++	7-8	88.89±0.8 a	+++	91.6±2.7
T_2	5-6	97.22±0.8 a	+++	5-7	94.44±0.8 a	+++	95.8±1.4
T_3	5-6	88.89±2.1 a	+++	5-6	86.11±0.8 a	+++	87.5±1.4
T_4	4-6	69.44±2.1 b	++	4-6	69.44±0.8 b	++	69.4±0.0
T_5	7-8	91.67±1.4 a	+++	7-8	88.89±2.1 a	+++	90.2±1.4
T_6	6-7	91.67±1.4 a	+++	7-8	91.67±1.4 a	+++	91.6±0.0
T_7	5-7	94.44±0.8 a	+++	6-7	94.44±0.8 a	+++	94.4±0.0
T_8	5-7	86.11±2.1 a	+++	5-7	83.33±2.4 a	+++	84.7±1.4
mean		88.23±3.08			86.15±2.8		

+ Slight callus, ++ Moderate callus, +++ Massive callus and same letter were not significantly different at 0.05 probability level using LSD. In each treatment, 36 explants were used.

3.3 Plantlet regeneration

The results indicate that, among different concentrations and combinations, treatment T_4 (0.5 mgl^{-1} BAP +0.1 mgl^{-1} IBA) showed better performance (Kalijira 91.67±0.18 and Chinigura 83.33±0.22) to produce plantlet while treatment T_2 (0.1 mgl^{-1} IBA) shows the lowest results (Kalijira 41.67% and Chinigura 41.33%). The range of shoot number (Kalijira 4-8 and Chinigura 3-8) and mean performance (Kalijira 6.63±0.18 and Chinigura 6.30±0.05) was also better for T_4 treatment and the lowest for T_2 (0.1 mgl^{-1} IBA). Overall, Kalijira was found to be more efficient in producing plantlets than that of Chinigura.

Another experiment has been performed to find out the effect of 2,4-D on plant regeneration. Calli obtain from different concentration of 2,4-D (i.e. 1.0, 2.0, 3.0 and 4.0 mgl^{-1}) were used for regeneration.

The result showed that the highest percentages of callus producing shoot (40%) were observed from the callus obtained from low concentration (Table 4). In addition, highest number of shoots was observed on the callus derived from 2.0 mgl^{-1} of 2,4-D treatment. However, callus induced from high concentration of 2, 4-D (3.0 mgl^{-1} or more) was found to be inefficient for plantlet regeneration.

Table 3: Regeneration efficiency of from callus derived from mature seeds (calli were obtained from 2.0 mgl^{-1} of 2,4-D)

	Kalijira				Chinigura			
Treatments	Shoot induction (%)	Number of shoot		(%) of shoots inducted root	Shoot induction (%)	Number of shoots		(%) of shoots inducted root
		Range	$\bar{X}$ ±SE			Range	$\bar{X}$ ±SE	
T_1	0	-	-	-	0	-	-	-
T_2	41.67±0.26 e	1-4	2.80±0.20 c	100	41.33±0.62 e	2-3	2.60±0.23 f	100
T_3	50.00±0.58 d	3-5	3.83±0.17 d	100	50.00±0.44 d	3-5	3.67±0.09 d	100
T_4	91.67±0.63 a	4-8	6.63±0.18 a	100	83.33±0.22 a	3-8	6.30±0.05 a	100
T_5	66.67±0.66 b	3-7	4.12±0.24 bcd	100	58.33±0.33 c	3-6	4.00±0.29 c	100
T_6	50.00±0.88 d	2-6	3.83±0.00 d	100	50.00±0.58 d	2-5	3.00±0.29 e	100
T_7	50.00±0.58 d	2-5	4.00±0.5 cd	100	41.67±0.55 e	2-5	3.20±0.11 e	100
T_8	66.67±0.41 b	3-8	4.37±0.25 b	100	66.67±1.20 b	3-8	4.75±0.07 b	100
T_9	58.33±1.01 c	3-7	4.28±0.42 bc	100	58.33±0.68 c	2-7	4.14±0.25 c	100
Mean (%)	59.37				56.25			

Treatments with the same letter were not significantly different at 0.05 probability level using LSD. In each treatment 12 explants were used.

Table 4: Plant regeneration efficiency of callus induced from different combinations of 2,4-D.

Calluses of different concentration of 2,4-D (mgl^{-1})	Concentration of BAP+IBA (mgl^{-1})	% of callus producing shoots	Average number of shoots per culture $\bar{X}$ ±SE	% of callus producing shoots	Average number of shoots per culture $\bar{X}$ ±SE
		Kalijira		Chinigura	
1.0	0.5+0.1	40	4.5±0.29	40	4.0±0.40
2.0	0.5+0.1	20	6.5±1.50	30	5.6±0.33
3.0	0.5+0.1	20	3.5±0.50	20	3.5±0.50
4.0	0.5+0.1	10	1.0±0.00	10	1.0±0.0

4 DISCUSSION

Despite the great importance of aromatic rice, little information is available on the callus induction and plant regeneration method through *in vitro* culture. The present study investigated the effect of various growth regulators on callus induction and plant regeneration efficiency in two Bangladeshi Traditional Aromatic Rice var. Kalijira and Chinigura.

4.1 Callus induction

The results showed that MS medium supplemented with 2 mgl^{-1} of 2,4-D was the most effective in callus induction for both cultivars Kalijira and Chinigura. This indicate that the use of 2,4-D with 2 mgl^{-1} was adequate for production of high amount of callus in rice. This finding is in

agreement with previous report by Rashid *et al.* (2003), where they showed that Basmati 370, Basmati 385 and KS 282 produced high amount of callus cultured on MS medium supplemented with 2.0 mgl^{-1} 2,4-D. Sikder *et al.* (2006) also reported that 2.0 mgl^{-1} 2,4-D is better for Chinigura callus induction. Similar results were also found in Thai aromatic rice KDML105 (Summart *et al*., 2008), ASD 16, ADT 43, Basmati 370, Pusa Basmati and Pokkali (Revathi and Pillai, 2011; Libin *et al*., 2012; Islam *et al*., 2005). Our results further revealed that the use of 2,4-D with cytokinin could be helpful for high and early production of callus. Similar observations were also reported in rice (Alam, 1994; Khondokar, 1999). Taken together, the findings from this study will be very useful for producing high frequency callus induction that is the prime step for crop improvement or rapid propagation through biotechnological approaches.

4.2 Plant regeneration

Among different concentrations and combinations, treatment T_4 (0.5 mgl^{-1} BAP +0.1 mgl^{-1} IBA) showed better performance to produce plantlets. The range of shoot number and mean performance was also found to be better for T_4 treatment. Similar results were reported on indica (Khanna and Raina, 1998) and Japonica rice cultivars (Lee et al., 2002). However, Sripichitt and Cheewasestatham (1994) reported that MS agar medium supplemented with 1 mgl^{-1} indol-3-acetic acid (IAA) and 4 mgl^{-1} benzyladenine (BA) induced highest percentage of calli forming shoots. Thadavong *et al.* (2002), Rashid *et al.* (2003), Sikder *et al.* (2006), Jubair *et al.* (2008) and Libin *et al.* (2012) also showed similar results. In our study, high concentrations of 2,4-D (2.0 mgl^{-1} or more) were found to be suitable for callus induction but these calli were not efficient for plant regeneration. In this study, results also showed that Kalijira is more efficient than Chinigura for producing plantlet from callus.

Our findings provide a simple *in vitro* protocol for generating high frequency callus formation and its subsequent regeneration for aromatic rice. These findings can also be manipulated for disease and pest resistant variety, stress and salt tolerance variety through tissue culture and gene transfer techniques.

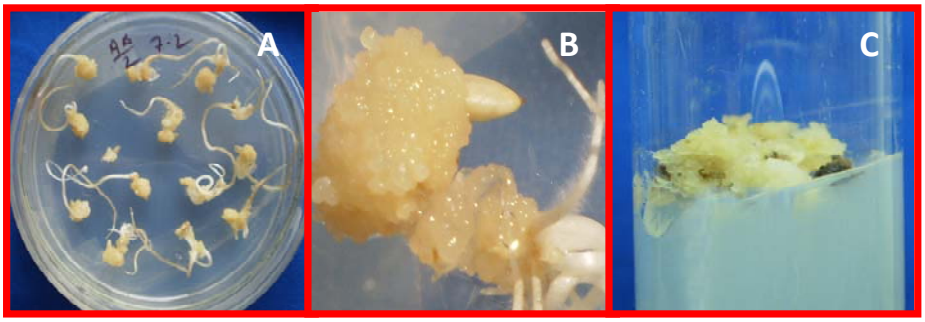

Figure 1: (A) Induction of callus from mature seeds in MS+2.0 mgl^{-1} of 2,4-D. (B) Highlight a single seed derived callus. (C) Proliferation of callus.

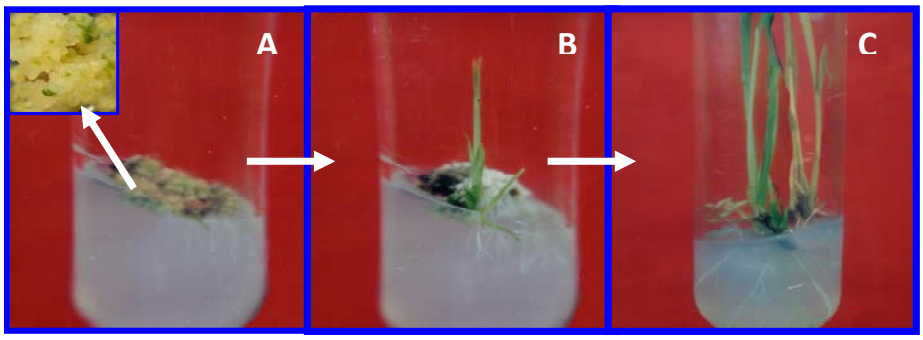

Figure 2: (A) Callus showing green spot on regeneration medium (MS +1.5 mgl^{-1} BAP +0.1 mgl^{-1} IBA). (B) Shoot formation from embryonic callus (MS+1.5 mgl^{-1} BAP+ 0.1 mgl^{-1} IBA). (C) Root proliferation of shoots in 1/2 MS without any growth regulators.

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