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Abstract

Manipulation of agronomic traits at the cellular and molecular levels offers an efficient approach to enhance conventional breeding efforts for rice improvement. Plant regeneration protocols, required for biotechnological applications, have not yet been developed for a number of important rice cultivars. This study was conducted to establish a system for plant regeneration of elite rice cultivars adapted to the southern U.S.A. Callus was induced from dehusked grains of cultivars Alan, Katy, and LaGrue, on MS media containing 0.5, 2, and 4 mg L^{-1} 2,4-D, with 0.5 mg L^{-1} kinetin or without kinetin. Plant regeneration was accomplished by transferring the callus to a hormone-free medium. Callus proliferation was influenced by 2,4-D, kinetin, and genotype in two-way interactions. The effects of these factors on embryogenesis and rhizogenesis was expressed in a three-way interaction. Depending upon the genotype up to 50% plant regeneration was obtained. In most cases treatments consisting of 0.5 to 2 mg L^{-1} 2,4-D plus 0.5 mg L^{-1} kinetin produced the best callus proliferations with the highest embryogenic capacity. Regenerants grew to maturity in soil and produced viable seeds. The establishment of this regeneration system is essential for the development of a genetic transformation system for the aforementioned commercial rice cultivars.

Introduction

Rice (*Oryza sativa* L.) is the staple food of more than half of the world population (Pathak, 1982). The importance of rice makes it a prime target for genetic manipulations through biotechnology. In general, the application of biotechnological approaches for crop improvement is limited by the availability of plant regeneration methods. Rice has been the focus of numerous studies aimed at inducing somatic embryogenesis that will lead to plant regeneration (Heyser et al., 1983; Raghava and Nabors, 1984; Oard and Rutger, 1988; Mirlohi et al., 1989; Tsukahara and Hirosawa, 1992a; Tsukahara and Hirosawa, 1992b; Rueb et al., 1994). Intraspecies 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). Our goal was to determine the regeneration requirements for commercially important rice cultivars adapted to the southern U.S.A., particularly Arkansas, the leading rice-producing state.

The current investigation is part of a strategy designed to develop transformation systems for Arkansas rice cultivars that will facilitate the introgression of transgenes conferring resistance to diseases and, ultimately, leading to the development of improved cultivars by enhancing conventional breeding programs. The objectives of this study were to 1) test the effects of 2,4-D and kinetin on callus induction from mature rice seeds, 2) evaluate the effect of callus induction treatments on subsequent plant regenerations, and 3) examine the genotypic responses of three commercial rice cultivars.

Materials and Methods

Seed Sterilization.--Seeds of rice cultivars 'LaGrue', 'Katy', and 'Alan' were obtained from the Arkansas Agricultural Experiment Station Rice Research and Extension Center, Stuttgart, Arkansas. The seeds were dehusked manually to preserve the embryos from mechanical damage. The dehusked seeds were surface sterilized in 70% ethanol for 1 min and then shaken for 30 mins on a gyratory shaker at 200 rpm in 2.6% w/v sodium hypochlorite (50% Clorox) containing 3 drops of Tween 20 per 100 ml Clorox solution. The seeds were rinsed in sterile distilled water and cultured on callus induction media.

Culture Medium.--The culture medium consisted of MS basal salts (Murashige and Skoog, 1962) containing 1 mg L^{-1} each of thiamine-HCl, pyridoxine-HCl, and nicotinic acid, 2 mg L^{-1} glycine, 100 mg L^{-1} inositol, 40 g L^{-1} sucrose, and 10 g L^{-1} agar [Agar-agar/Gum agar] (Sigma Chem. Co., St. Louis). The medium was adjusted to pH 5.8 with 1N KOH, autoclaved, and dispensed in 100-mm x 15-mm petri dishes. Callus induction medium was supplemented with 2,4-dichlorophenoxyacetic acid (2,4-D) (Sigma) at 0.5, 2, or 4 mg L^{-1} and fufurylaminopurine

(kinetin) (Sigma) at 0 or 0.5 mg L^{-1}. Plant regeneration media contained no growth regulators.

Culture Stages and Conditions.--The seed cultures were incubated at 24° C under a 12-h photoperiod of cool-white fluorescent light (40 $\mu Em^{-2}s^{-1}$). After 4 weeks, calli were separated from the seed explants and transferred to a fresh callus induction medium and incubated for an additional 4 weeks. Calli were then weighed and transferred to plant regeneration medium. After 10 weeks, the number of embryogenic calli, with the capacity to regenerate plants, and the number of rhizogenic calli, with the capacity to form only roots, were determined.

Plant Establishment.--When plantlets were approximately 10 mm long, they were separated and transferred from the petri plates to a hormone-free medium dispensed in 150-mm x 25-mm tubes to allow for elongation. After 2 to 3 weeks, agar was washed from the root regions and the plantlets were transplanted to a potting mix (Redi-Earth Peat-Lite Mix, Grace-Sierra Hort. Products Co., Mipitas, CA), watered with half-strength MS salts, misted with water to maintain humidity, placed in clear plastic containers and maintained under the same cultural conditions as prior to transplanting. The humidity was gradually reduced by increasing the opening of the container over a period of 2 weeks. The plants were then relocated to a greenhouse and grown to maturity.

Statistical Analysis.--The experiment was set up as a completely randomized three-factor factorial design, 3x3x2. The factors tested were cultivar (three genotypes), 2,4-D (three concentrations), and kinetin (two concentrations). Data were subjected to analysis of variance (ANOVA) based on 20 replications for mean callus weight and 10 to 20 replications for percentage of morphogenesis, plant regeneration and root formation. Transformation of the percentage data was not necessary. The means were separated, where appropriate, at the 5% significance level using the least significant difference (LSD) for the callus weight and a multiple t-test for the percentage of morphogenesis.

Results and Discussion

Callus Induction.--Callus formation was observed within 2 weeks of culturing. Seed germination often preceded callusing on media containing the lowest 2,4-D concentration, but on either of the higher 2,4-D concentrations, callus formed directly (Fig. 1). While the percent of callusing (90 to 100%) was not influenced by the experimental factors, callus fresh weight was significantly affected by the plant genotype and the concentrations of 2,4-D and kinetin expressed in the two-way interactions illustrated by the ANOVA (Table 1). Table 2 shows the means associated with each of these three two-way interactions. The top section of Table2 represents the cultivar/kinetin interaction, the middle section describes the cultivar/2,4-D interaction, and the bottom section illustrates the kinetin/2,4-D interactions.

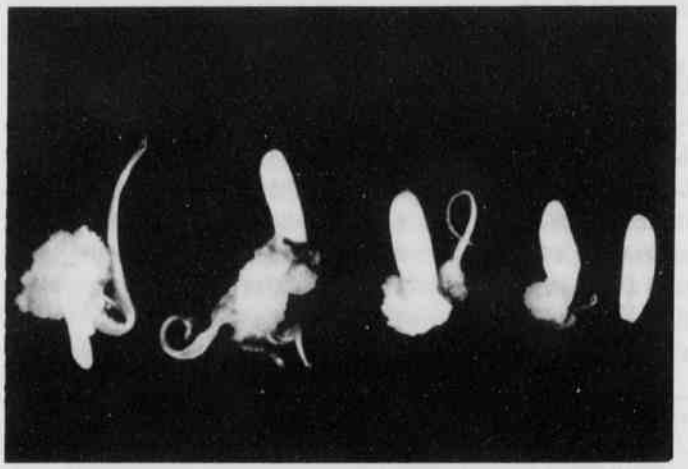

Fig. 1. Successive stages of callus induction from rice mature seeds.

Callus proliferation differed significantly among cultivars and depended upon the concentration of 2,4-D and the concentration of kinetin in a two-way interaction determined by the ANOVA (Table 1). On kinetin-free medium there was no significant difference between Katy and LaGrue, but Alan produced significantly larger callus (Table 2). With 0.5 mg L^{-1} kinetin, however, callus proliferation among cultivars differed significantly. Alan produced the largest callus, followed by LaGrue, and then Katy.

Kinetin had no significant effect on callus weight of Alan and Katy. However, a significant increase of callus weight of LaGrue was associated with the addition of 0.5 mg L^{-1} kinetin to the callus medium (Table 2).

The concentration of 2,4-D significantly influenced callus fresh weight (Table 2). In general, as the concentration of 2,4-D increased, callus growth decreased at a rate that was cultivar-specific, hence the two-way interaction. Significant decreases in callus weight occurred in all three cultivars when the concentration of 2,4-D was increased from 0.5 mg L^{-1}, the level at which the greatest callus growth was achieved, to 2 mg L^{-1} (Table 2). Although, an increase in 2,4-D from 2 mg L^{-1} to 4 mg L^{-1} did not cause a significant difference in callus growth for Katy or LaGrue, it caused a significant reduction in callus weight for Alan. On media supplemented with 0.5 mg L^{-1} 2,4-D, mean callus weights for the three cultivars differed significantly. With 2 mg L^{-1} only Katy produced signifi-

Table 1. ANOVA of callus weight, percentage embryogenesis, and percentage rhizogenesis of callus induced from mature rice seed explants.

Source	DF	Callus Weight		% Plant Regeneration		% Root Formation	
		Mean square	P-value	Mean square	P-value	Mean square	P-value
Cultivar	2	69284	0.0001*	8123	0.0012*	4465	0.1155
Kinetin level	1	18	0.9493	7919	0.0102*	535	0.6100
2,4-D level	2	562107	0.0001*	1947	0.1942	1537	0.4737
Cultivar x Kinetin	2	23307	0.0060*	29	0.9755	9680	0.0097*
Cultivar x 2,4-D	4	42480	0.0001*	4168	0.0080*	6532	0.0141*
Kinetin x 2,4-D	2	31666	0.0010*	2268	0.1486	459	0.7996
Cult x Kin x 2,4-D	4	10451	0.0562	3198	0.0308*	21121	0.0001*
Error, callus	342	4494	-	-	-	-	-
Error, plant or root	258	-	-	1180	-	2052	-

*Significant at p = 0.05

cantly less callus than the other two cultivars, but on 4 mg L^{-1} all three cultivars were similar (Table 2).

Table 2. Effects of 2,4-D, and kinetin levels on callus induction from mature seeds of three rice cultivars.

	Mean Callus Weight (mg)		
	Cultivar		
Kinetin (mg L^{-1})	Katy	LaGrue	Alan
0.0	129c[1]	136c	189a
0.5	130c	161b	178a
2,4-D (mg L^{-1})	Katy	LaGrue	Alan
0.5	169c[2]	222b	289a
2.0	117e	120de	149cd
4.0	102e	104e	112e
	2,4-D (mg L^{-1})		
Kinetin (mg L^{-1})	0.5	2.0	4.0
0.0	202b[3]	135c	116c
0,5	251a	122c	95d

Means are based on 20 calli per hormonal treatment.
Means within a group followed by the same letter do not differ significantly.
[1]LSD (0,05) = 24; [2]LSD (0.05) = 30; [3]LSD (0.05) = 24.

Based on the ANOVA of callus weight (Table 2), the influence of the hormones was dependent upon the concentration of both hormones, thus the two-way interaction between 2,4-D and kinetin. At 0.5 mg L^{-1} 2,4-D, the addition of kinetin significantly stimulated callus growth for all three cultivars (Table 2). As the concentration of 2,4-D increased to 2 mg L^{-1} callus growth was insensitive to the addition of kinetin, but when the level of 2,4-D reached 4 mg L^{-1} callus proliferation was significantly inhibited by the addition of 0.5 mg L^{-1} kinetin (Table 2).

Somatic Embryogenesis and Rhizogenesis.--When callus tissues were transferred to regeneration media, some exhibited no response or became necrotic, but the majority developed either roots (rhizogenic calli), or plantlets (embryogenic calli). Plant regeneration (Fig. 2) was observed as early as 3 weeks after calli were placed on regeneration medium, especially from calli that had formed on the lowest level of 2,4-D (0.5 mg L^{-1}). 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. This variability was influenced by the callus-induction treatments, i.e. kinetin and 2,4-D combinations, hence the three-way interaction (Table 1).

Upon transfer to the hormone-free regeneration medium, calli induced on 0.5 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin resulted in the highest plant regeneration percentage for both Katy and Alan cultivars (Table 3). At this concentration of 2,4-D, the omission of kinetin from the callus induction medium did not significantly alter the regeneration percentage for Alan but significantly inhibited regeneration for Katy. The highest percentage of regeneration obtained for LaGrue was from calli induced on 2 mg L^{-1} 2,4-D and 0.5 mg L^{-1} kinetin. At this level of 2,4-D, omitting kinetin also significantly reduced the percentage of plant regeneration for LaGrue.

Table 3. Effects of 2,4-D and kinetin concentrations in the callus induction medium on subsequent plant regeneration and root formation from calli induced from rice seed explants representing three cultivars.

	2,4-D Concentration (mg L^{-1})					
	0.5	2.0	4.0	0.5	2.0	4.0
Cultivar	0.0 mg L^{-1} Kinetin			0.5 mg L^{-1} Kinetin		
	% Plant Regeneration [1]					
Katy	7bA[2]	17bB	0bA	46aA	0bB	14bA
LaGrue	25bA	33bA	6cA	13bA	50aA	33bA
Alan	11aA	0bB	0bA	24aA	0bB	17aA
	% Root Formation[1]					
Katy	86aA[2]	67aA	57aB	54aA	33bB	43aA
LaGrue	75aA	67aA	6bC	40aA	50aB	67aA
Alan	28bB	50bA	93aA	71bA	94aA	50bA

[1]Percentage is based upon 10 to 20 replications per hormonal combination.
[2]Means followed by the same letter, low case within a row and capital within a column, do not differ significantly based upon multiple t-test at 5% significance level.

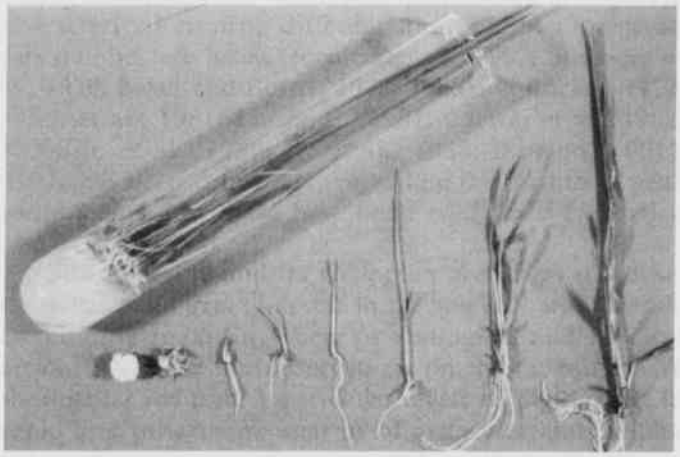

Fig. 2. Successive stages of plant regeneration and growth from rice seed-derived callus.

A comparison of the regeneration percentages (shown by columns in Table 3) revealed that the three cultivars did not differ significantly for calli induced on 0.5 mg L^{-1} 2,4-D, regardless of the kinetin concentration; the same was true for 4 mg L^{-1}, but they did differ significantly at the 2 mg L^{-1} level.

In contrast to callus induction data where Alan produced the largest callus (Table 2), overall, the percentage of plant regeneration was the lowest (average of 9% for all treatments) for this genotype (Table 3). Katy and LaGrue produced a total average of 14% and 27% regeneration, respectively (Table 3).

Generally, rhizogenesis was inversely related to the percentage of plant regeneration (Table 3). However, this relationship did not hold for all the treatments. For example, calli induced from LaGrue on 4 mg L^{-1} 2,4-D produced 6% embryogenesis and rhizogenesis.

The percentage of rhizogenesis among the cultivars was highly variable. Rhizogenesis in Katy was the least from calli induced on 2 mg L^{-1} 2,4-D in the presence of kinetin. All other treatments resulted in similar amounts of rhizogenesis (Table 3). LaGrue produced the least rhizogenesis from calli induced on 4 mg L^{-1} 2,4-D, while all other treatments resulted in similar amounts of root-producing calli. Rhizogenesis in Alan was highest on 4 mg L^{-1} 2,4-D in the absence of kinetin and on 2 mg L^{-1} 2,4-D in the present of kinetin. The other treatments had similar effects (Table 3).

Plant Establishment.--Eighty five to 100% regenerated plantlets survived in soil regardless of cultivar or in vitro treatments (Fig. 3). Under greenhouse conditions, the regenerants exhibited normal growth and produced viable seeds.

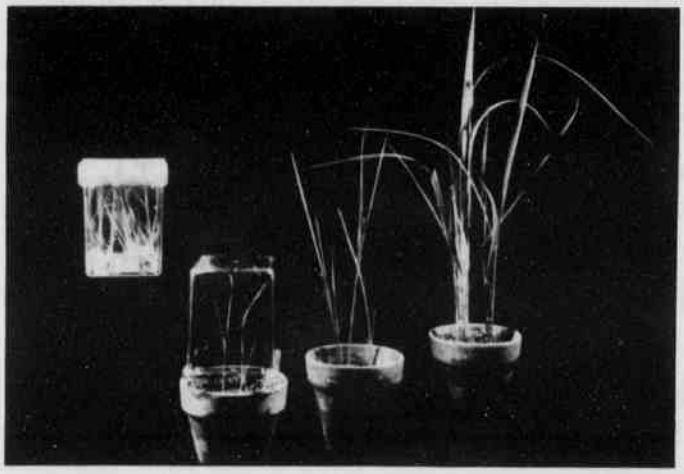

Fig. 3. Regenerants transplanted to soil exhibiting normal appearance.

Conclusions

A plant regeneration system for three commercial rice cultivars has been established using mature seed as a source for explants. The system consists of two stages, callus induction and plant regeneration. A callus induction medium suitable for all three cultivars consisted of MS medium containing 0.5 mg/L 2,4-D and 0.5 mg/L

kinetin. Transfer of calli induced by this treatment to a hormone-free medium resulted in callus redifferentiation into somatic embryos that germinated into plantlets. Although increasing the 2,4-D level to 2 mg/L 2,4-D resulted in higher percentages of regenerative calli for LaGrue, in general, the reduced 2,4-D concentration in the callus induction medium expedited subsequent plant regeneration. For long-term callus maintenance, however, concentrations of 2,4-D higher than 0.5 mg/L would be more appropriate to suppress unwanted redifferentiation.

In summary, optimum callus growth was obtained on the lowest level of 2,4-D. The addition of kinetin to the callus induction medium modified callus growth based on varying 2,4-D concentrations and plant genotype. Kinetin enhanced the regeneration capacity of callus induced from Katy and LaGrue but made no difference for Alan. This investigation was a critical step in the development of a regeneration system for the application of biotechnological approaches to Arkansas rice improvement. This highly efficient, reproducible system will be used to develop genetic transformation techniques for these important rice cultivars. Another potential application of this regeneration system is the development of plants with desirable agronomic characteristics from in-vitro-selected mutant cell lines. To our knowledge there is no previous report on the regeneration of these rice cultivars.

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