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Development of *in vitro* Regeneration Protocols for Arkansas Rice Varieties (*Oryza sativa L.*)

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Abstract.—Efficient regeneration of *in vitro* cultures of *Oryza sativa L.* is essential for successful manipulation of recombinant DNA technologies. Arkansas rice varieties perform better on modified Gamborg (B5) media, than on Murashige and Skoog (MS) or N6 media, which are more frequently reported in the literature. While 2,4-dichlorophenoxyacetic acid (2,4-D) is commonly used for regeneration treatments of rice, Picloram (Pic) provides a possible alternative as a synthetic auxin source. Although regeneration frequency appears low, complete regeneration (whole plantlets) was faster and development of the shoot was superior on picloram treatments as compared to 2,4-D.

Screening 5 Arkansas varieties for regeneration efficiency on 3 treatments, 2 drawn from the literature and 1 based on Pic, shows distinct rankings for successful identification of totipotent established lines. LaGrue, important for both production and breeding purposes, shows the highest ranking for successful regeneration as well as for uniform response of individual seedling lines across regeneration treatments. Other varieties proved unsuitable for transformation work due to lack of regeneration response and/or non-uniform response to regeneration treatments. Identifying these parameters will facilitate the ultimate transfer of recombinant DNA into tropical *Japonica* rice types grown in Arkansas for trait improvement or gene expression studies.

Key words:— in vitro, Oryza sativa L., DNA, Arkansas rice, Gamborg (B5), Murashige and Skoog (MS), N6 media, 2,4-dichlorophenoxyacetic acid, Picloram (Pic), synthetic auxin, *Japonica.*

Introduction

Transformation technologies currently use MS (Murashige and Skoog 1962) or N6 (Chu 1978) media treatments (Rashid et al. *1*996, Toki 1997) with several choices of plant growth regulators (PGRs) such as 6-benzylaminopurine (BA) + α-naphthaleneacetic acid (NAA) or kinetin (Kin) + NAA. While the use of picloram as an auxin is not reported for rice regeneration, it has been successfully used for other monocots (Phillips and Luteyn 1983). Rice varieties previously studied vary widely, and includes *O. sativa* ssp. *Japonica* as well as ssp. *Indica* and *Javonica*. Our interest is with the tropical *Japonica* types important for Arkansas rice production. High regeneration efficiency is necessary for successful transformation of rice (Toki 1997). Identification of highly efficient regenerator varieties could provide uniformity in such systems. While 2,4-D is commonly used for short term culture and regeneration of rice, long term exposure may ultimately suppress regeneration potential and make it difficult to utilize known regenerator lines, thus requiring longer culture time. Picloram, which can be used for other monocots, may provide a suitable substitute for 2,4-D and avoid this problem (Dode et al. 2000). Technology directly applicable to local varieties is critical for ultimate success in improving rice yield in Arkansas.

Material and Methods

Five rice varieties were provided by the University of Arkansas Rice Research and Education Center (RREC) at Stuttgart, Arkansas, courtesy of Dr. Karen Moldenhauer: Drew, Gulfmont, Katy, LaGrue and Mars. All varieties were tested for callus induction. De-hulled rice seed were surface sterilized by exposure to 50% Clorox and then plated onto initiation media as the standard starting material for all experiments (Fig. 1). Cultures were placed in dark incubators at 28°C for initial growth. Calli were incubated in constant light conditions only when transferred to regeneration treatments. Calli 1-60 days old were grown in 60 x 15 mm Petri dishes, all regeneration treatments of larger tissues were grown in 100 x 15 mm Petri dishes. Preliminary experiments were conducted to compare modified Gamborg B5 media (Dunstan and Short 1977) with MS and N6 media as the basal salts and nutrients formulation for rice culture.

Rice calli were grown for 60 days on initiation media using either 2,4-D or Pic (2.2 mg/l and 5 mg/l, respectively). Residual tissues from the rice seed were removed after 30 days, and calli were transferred at 60 days to regeneration treatments to allow for accumulation of biomass. Regeneration treatments labeled

Fig. 1. Callus formation and development on three different basal salts using Katy: A) N6, B) B5, and C) MS.

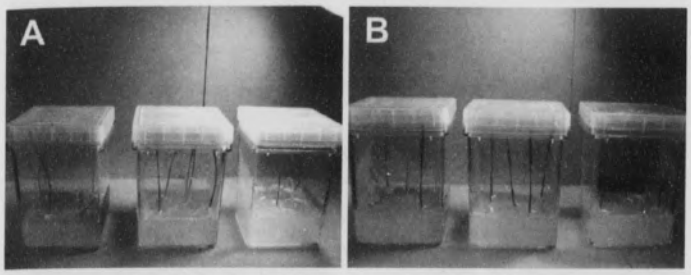

Fig. 2. Seed germination and plant development of two varieties on three different basal salts. A) Katy: MS, B5, and N6 (left to right). B) Mars: MS, B5 and N6 (left to right).

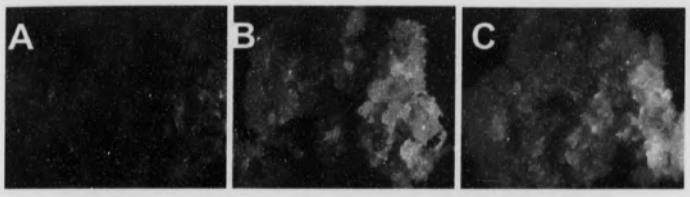

Fig. 3. Callus morphology after 60 days incubation varied on basal salts treatments: A) N6, B) MS, and C) B5.

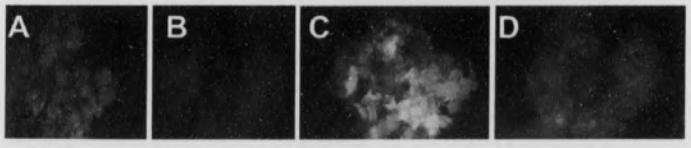

Fig. 4. Callus morphology after 60 days incubation varied on 2,4-D (A, B), and Picloram (C, D) treatments.

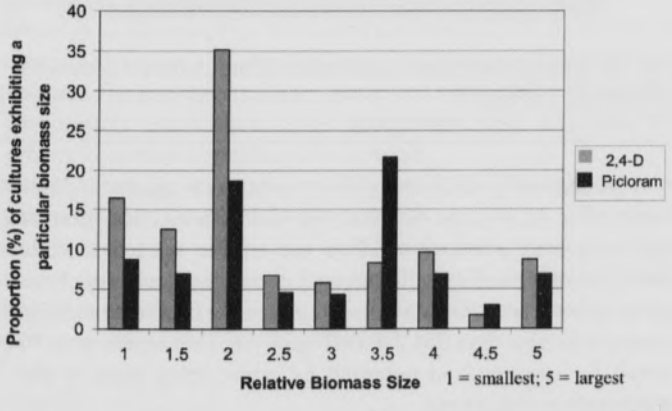

Fig. 5. Biomass comparison using 2,4-D and Picloram. A relative scale using 1 = smallest biomass and 5 = largest biomass was utilized to categorize distribution of cultures within treatments.

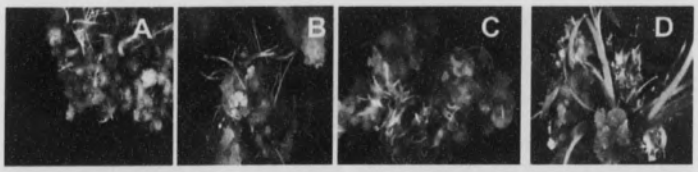

Fig. 6. Somatic embryogenesis response using 2,4-D ranged from embryogenic callus formation with incomplete formation of shoot apices (A, B, C) to whole plant formation (D).

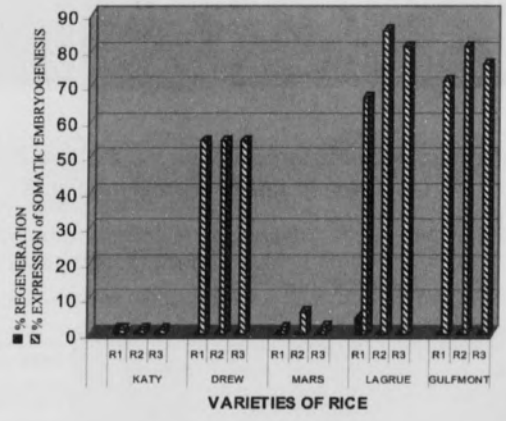

Fig. 7. Induction of somatic embryogenesis and whole plant regeneration of 5 rice varieties on R1, R2, and R3 PGR treatments following callus proliferation on 2,4-D.

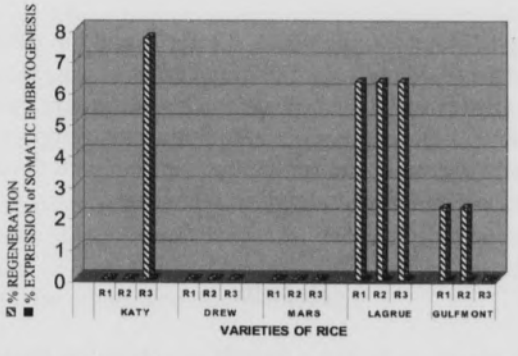

Fig. 8. Induction of somatic embryogenesis and whole plant regeneration of 5 rice varieties on R1, R2, and R3 PGR treatments following callus proliferation on Picloram.

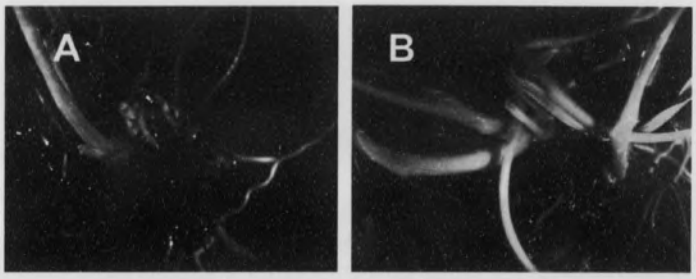

Fig. 9. Somatic embryogenesis response using Picloram leading directly to whole plant formation (A, B).

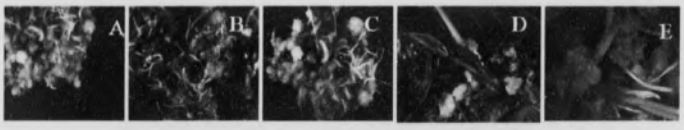

Fig. 10. Embryogenic callus formation on R1, R2, and R3 PGR treatments (A, B, C). Complete plant regeneration on R1 PGR treatment (D, E).

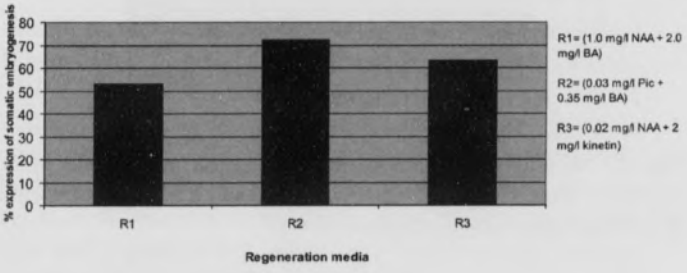

Fig. 11. Comparison of R1, R2, and R3 PGR treatments for expression of somatic embryogenesis.

R1 (1.0 mg/l NAA + 2.0 mg/l BA), R2 (0.03 mg/l Pic + 0.35 mg /l BA), and R3 (0.02 mg/l NAA + 2 mg/l kinetin) were tested for 2 months. Observations were made weekly, data were collected every 30 days. Lines were scored for frequency of induction of somatic embryogenesis and for the frequency of well developed plantlets with both apical and radicle meristems.

Results

Initial studies indicated a benefit of comparing basal salts formulations. Early experiments indicated that growth potential of rice *in vitro* cultures could be optimized by using modified Gamborg B5 media, in comparison to MS and N6 media (Fig. 2).

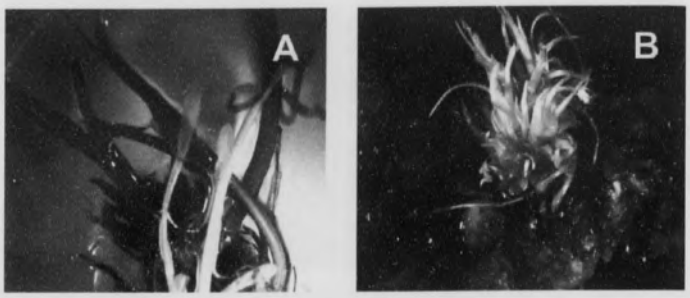

Fig. 12. Regeneration of albino shoots from Gulfmont (A) and LaGrue (B) cultures.

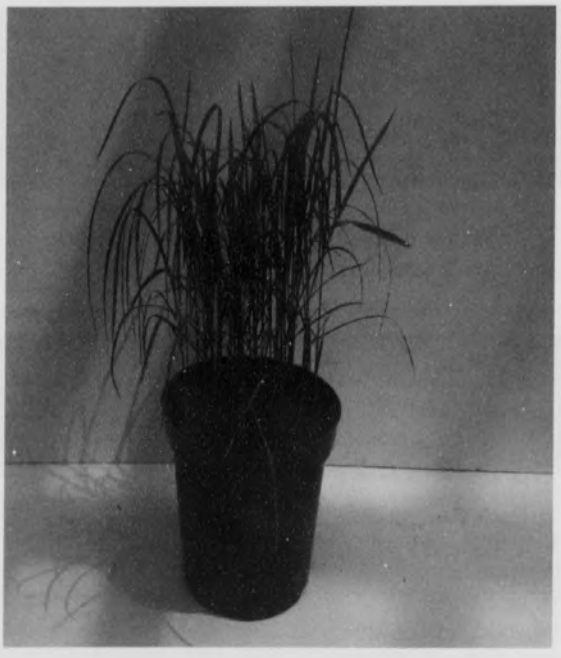

Fig. 13. Established plant regenerated from cultured somatic embryo of LaGrue.

Dramatically different callus types grew on the induction media (Fig. 3). The use of 2,4-D resulted in small, more compact, and nodulated phenotypes. The use of Pic tended to induce prolific rooting and only higher concentrations gave more typical callus phenotypes. Over time, Pic generally produced calli with greater biomass than did 2,4-D (Fig. 4, 5). These calli were then tested for regeneration potential by transferring them to the 3 regeneration treatments.

When the rice varieties were scored for regeneration potential, it was clear that the PGRs used during callus formation are important. Calli originating on 2,4-D showed high levels of embryogenic induction (Fig. 6, Table 1), up to 89% for LaGrue (Fig. 7).The 5 varieties, ranked from highest to lowest, were LaGrue, Gulfmont, Drew, Mars, and Katy (Table 1). Whole

Table 1. Number of individual seedling-derived rice callus lines responding to different regeneration treatments (R1, R2, or R3 PGR treatments). Sample size of approximately 50 explants per variety per regeneration treatment. R1 (1.0 mg/l NAA + 2.0 mg/l BA), R2 (0.03 mg/l Pic + 0.35 mg /l BA), and R3 (0.02 mg /l NAA + 2 mg/l kinetin).

	Varieties				
Response to three varying regeneration treatments	**Gulfmont**	**LaGrue**	**Drew**	**Katy**	**Mars**
R1, R2, and R3	14	12	4	0	4
R1 only	0	0	1	0	1
R2 only	1	0	1	2	1
R3 only	0	1	0	1	1
R1 and R2	1	1	0	3	0
R2 and R3	1	2	3	3	0
R1 and R3	0	0	0	0	0

plant regeneration at 60 days on 2,4-D was limited to LaGrue and Katy, and was low (4.8% for LaGrue and 1.4% for Katy; Fig. 7). Calli induced on Pic showed higher whole plant regeneration (up to 7.7%; Fig. 8). Although whole plant regeneration was faster using Pic (30 days compared to 60 days on 2,4-D), sustained somatic embryogenesis was impossible because the embryogenic callus did not proliferate and callus quality declined (Fig. 9). Complete plant regeneration occurred only on R1 from 2,4-D-derived cultures at 60 days (Fig. 10D). All treatments supported expression of the embryogenic callus; however, R2 gave the highest frequency of somatic embryo development, while R1 showed the lowest frequency of embryogenic callus expression (53.1% on R1, 72.15% on R2, and 63.29% on R3; Fig. 10, 11). Whole plant regeneration within 30-60 days after induction sometimes gave abnormal phenotypes. Albino regenerants were found in Gulfmont (Fig. 12A) and LaGrue (Fig. 12B) and are of concern since such events will negatively impact transformation success. Despite this problem, numerous regenerated plants were successfully established in the greenhouse (Fig. 13) and were grown to maturity, yielding fertile seeds.

In a preliminary experiment we compared regeneration potentials of LaGrue with Nipponbare and Taipei 309, which are the two most often-cited varieties for rice regeneration and transformation (Toki 1997, Dong et al. 1996). Our initial results suggested that LaGrue was comparable to Nipponbare, but Taipei 309 was a better regeneration variety.

Conclusions

B5 basal media gave consistently better responses than either MS or N6.

Picloram produced higher biomass during callus induction events.

Although 2,4-D showed the better induction of somatic embryogenesis, complete regeneration events were fewer than with picloram treatments.

Picloram tended to regenerate whole plants faster (30 days vs. 60 days) than did 2,4-D.

LaGrue and Gulfmont were superior in regeneration response compared to the other varieties (Drew, Katy, and Mars) tested.

Inconsistent regeneration responses among callus lines of some varieties (Katy and Mars) make these varieties undesirable for transformation of unscreened lines.

Future Goals

Optimization of *in vitro* technology for rice for such variables as basal salt formulations, PGRs, and identification of efficient regenerator varieties will facilitate successful transformation protocols. Improving these parameters will facilitate the use of recombinant DNA technology in rice genetics. These improvements will ultimately make these tools useful for studying RNAi genetic manipulation techniques to target inhibition of lipoxygenase gene(s) expressed specifically in the rice grain, which will permit testing whether lipoxygenase enzyme activities lead to grain degradation during storage.

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