In Vitro Production of Axenic Seedlings in Rice: Effect of Sterilant and Media on Seed Germination Efficiency

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ABSTRACT

The effects of media and sterilization agents on *in vitro* germination of rice seeds (*Oryza sativa* L.) in two indica rice varieties (PAU 201 and PR 116) were studied. Out of two basal media formulations tested for seed germination, full strength MS basal medium supported the higher percentage of seed germination (76.59%) than half-strength basal medium (73.32%). The variety PR 116 exhibited the mean germination percentage of 76.66% in both the media. Among the sterilization methods studied, the percent seed germination and contamination varied with the sterilizing agent and duration of treatment. Of the three sterilization methods used Bavistin 1.0% + $HgCl_2$ 0.1% gave the best surface disinfection with minimum contamination of seed (0 to 2 %) and germination as good as (75 to 80%) when treatment duration was 6 minutes in both the rice cultivars. Though $HgCl_2$ (0.1%) with 8-10 min duration gave the better results regarding decontamination, but this was still not efficient as the residual of $HgCl_2$, specifically, mercury, suppressed the germination of rice seeds.

Key words: *Oryza sativa* L., *In vitro* germination, aseptic seedlings, sterilization, soaking duration

INTRODUCTION

Plant tissue culture techniques provide an alternative means of improvement obtaining somaclone induced variants, somatic hybridization and doubled haploids to develop inbred lines or for introducing genes of interest against insects and different diseases through genetic engineering. The successful application of *in vitro* methodologies for improvement of crop plants is mainly dependent on a

reliable and reproducible regeneration system.

An important problem encountered during tissue culture experiments is that field grown materials used as a source of explant, are heavily contaminated with organisms (fungi and bacteria) which are difficult to remove prior to culture. The *in vitro* environment in which the plant material is grown is also ideal for the proliferation of microorganisms and in most cases the microorganisms outgrow the plant tissues, resulting in the death of plant tissue. Later aseptic technique to develop the experimental culture techniques using young tissues from seeds germinated *in vitro* is absolutely necessary for the successful establishment and maintenance of plant cell, tissue and organ cultures. This technique has the advantage of providing very clean material as well as producing juvenile tissues which usually respond well in culture. There are four approaches in vogue for decontamination of explants viz. decontamination of seeds (Lindsey, 1967), aseptic removal of seeds from fruits (White, 1943), aseptic removal of embryos from seeds (Miflin, 1969) and direct decontamination of explants taken from mature plants (Baker and Phillips, 1962). Further more, for successful *in vitro* culture sterilization techniques is very important. The sterilization of explants and seeds can be done with different concentrations of various chemical components (sodium hypochlorite, calcium hypochlorite, mercuric chloride, silver nitrate etc.), fungicides, antibiotics, biocides and temperature applications (Gamborg and Philips, 1995). In sterilization procedures, both the concentration and the duration of exposure to disinfectants are important. If the concentration of sterilants is too high or the duration of exposure is too long, the plant tissues will be damaged and can even lead to non viability of seeds. Since the substances used for sterilization may have a negative effect on germination so sterilization methods are determined through investigations (Gamborg and Philips, 1995; George, 1993). A major difficulty in achieving axenic culture is the development of a sterilization method rigorous enough to kill contaminant bacteria and fungi, while being harmless to plant tissue. The objective of this study was to optimize media and sterilization methods i.e. sterilization chemicals and duration of treatment and their effect on seed germination/ decontamination in rice to produce axenic seedlings.

MATERIAL AND METHODS

Plant Material

The plant material comprised seeds of two varieties of rice viz. PAU 201 and PR 116 collected form Rice Section, Department of Plant Breeding and Genetics, PAU, Ludhiana from the harvest of 2007-08. Seeds of above cultivars were used as source

material and were thoroughly washed five times with tap water to remove the dust and debries completely, left in a beaker of water for few minutes, after which those floating on the water surface were discarded. Plump and mature seeds were chosen and washed in a solution containing a few drops of Tween 20 to which water was added, vigorously shaken and then thoroughly washed thrice by autoclaved double distilled water.

Basal media for germination of seeds

Experiments with two cultivars were carried out to obtain high frequency seed germination. The pH of the media was adjusted to 5.8 before autoclaving. Medium was then autoclaved at 121°C temperature and 15lb pressure for 20 min. Then medium was poured on autoclaved petriplate under laminar air flow. After surface sterilization 80-100 seeds of each cultivar were inoculated on Petri plates containing autoclaved media of seeds. These were incubated for 5 days at 28±2°C with a 16 h photoperiod provided by cool white fluorescent tubes. Germination was recorded after one week.

Media used

Full strength and half strength MS (Murashige and Skoog, 1962) media supplemented with 3% Sucrose and 0.8% agar were used for seed germination.

Sterilizing Agents and Disinfection Methods

Three surface sterilizing agents *viz.* mercuric chloride (0.1%), bavistin (1.0%) and mercuric chloride (0.1%) + Bavistin (1.0%) with different durations were used in the study. Seeds of two rice varieties viz. PAU 201 and PR 116 were disinfected to investigate the effect of type and duration of sterilant on seed germination.

After surface disinfection, 80-100 seeds from each treatment were placed on sterilization petri plates containing MS media. This was repeated three times. Eight to ten seeds were placed in each petri plates containing MS (Murashige and Skoog, 1962). The seed plates were kept in the dark for germination at 28 ± 2°C for 5 days. To prevent the air born inoculum of microbes, all sterilization work was performed in a laminar airflow cabinet as described by Kreider (1968). Upon removal from incubation, the seed germination and contamination was observed at 3 days intervals with minimal handling of the plates to reduce the contamination introduced by the air currents. Seed was considered to have germinated when the radical clearly emerged. Contamination was determined by visual inspection for fungal and / or bacterial growth and plates which contained microbial colonies were considered contaminated. Both seed germination and contamination were

recorded as percentage of total number of seeds cultured.

Statistical analysis was done according to the CPCS-I package using factorial CRD design. CD values at 5% level of significance were calculated and the interpretations were made accordingly.

RESULTS AND DISCUSSION

Results obtained for effect of surface sterilant and media on seed germination of two indica rice cultivars viz. PAU 201 and PR 116 used in this study have been described as:

Effect of basal media on germination of seeds

Out of two basal media formulations tested for seed germination, full strength MS salts and vitamins supported the higher mean percentage of seed germination (76.59%) than half-strength MS salts and vitamins (73.32 %). Analysis of variance revealed significant differences between varieties with respect to germination in both the media (Table 1). Maximum percent seed germination of 77.6% was observed for variety PR116 on MS full strength medium. The variety PR 116 also exhibited the higher mean germination percentage (76.66%) in both the media than PAU 201 (71.76%). The percent germination in half strength MS medium was 70.93 and 75.72% for PAU 201 and PR116 varieties respectively. So out of the two genotypes, screened for *in vitro* germination on two media variety PR 116 exhibited sufficiently high germination percentage (>75%) and was found as potential genotypes for further *in vitro* regeneration and transformation studies. Earlier workers (Al-Khayri *et al.*, 2001; Meneses *et al.*, 2005; Hoque *et al.*, 2007; Summart *et al.*, 2008; Ikram-ul-Haq *et al.*, 2010; Shahsavari *et al.*, 2010) have also reported MS full strength and MS half strength media for *in vitro* germination of rice seeds. Besides, some worker (Wenzhong *et al.*, 1994; Mariani *et al.*, 2003; Afolabi *et al.*, 2008 and Rahman *et al.*, 2010) have documented the use of MS

Table1. Effect of different media on seed germination of rice after 1 week

	Germination%		
Media/Varieties	**MS**	**½ MS**	**Mean**
PAU 201	72.59 (58.42)	70.93 (57.37)	71.76 (57.90)
PR 116	77.60 (61.75)	75.72 (60.48)	76.66 (61.11)
Mean	76.59 (60.08)	73.32 (58.92)	
CD at (.05) Varieties 3.08;		Media NS	

The values in parantheses are arcsin transformed values

full strength and/or MS half strength salts supplemented with B5 vitamins (Gomberg *et al*., 1968) media for *in vitro* germination of rice seeds.

Effect of Sterilizing Agents and Disinfection Methods

Rice seeds from the field are highly contaminated as they contain large numbers of bacteria and fungal spores. For any tissue culture study, the surface of explants must be fully sterilized or source of explant must be raised axenically *in vitro*. Thus, this experiment involved the establishment of suitable sterilization regimes for the rice seed to produce axenic seedlings. As a large percentage of the contamination was of fungal origin, inclusion of Bavistin in the pretreatment was essential for reducing the percentage contamination of the seedlings. To obtain the best explants, three seed sterilization methods (bavistin, mercuric chloride and combination of both) were compared for efficiency as sterilizing agents and subsequent effect on seed germination in present study. Eighty to hundred seeds of two *indica* rice varieties viz. PAU 201 and PR 116 (showing high germination (>70%) in earlier experiment were sterilized by the three methods as described in materials and methods with three replications. Table 2 summarizes the efficiency of the different seed surface sterilants, treatment duration and their effect on per cent seed germination and contamination in rice after 1 week. Among the sterilization methods studied, the percent seed germination and contamination varied with the sterilizing agent and duration of treatment. It was observed that with increase in duration of treatment from 4 to 10 minutes contamination was significantly reduced in all the three treatments. However, germination percentage also drastically decreased as treatment duration exceed beyond 6 minutes. In earlier studies, different sterilization methods have been reported to sterilize rice seeds (Chun *et al*., 1993; Kant *et al*., 2007; Karthikeyan *et al*., 2008). Of the three sterilization methods used in this study method 3 i.e. (bavistin 1.0% + $HgCl_2$ 0.1%) gave the best surface disinfection with minimum contamination of seed (0 to 2 %) and germination as good as (75 to 80%) when treatment duration was 6 minutes in both cultivars. Methods 1 and 2 did not give perfect sterilization because the extent of contamination was quite high (2 to 7.5%) even when treatment duration was enhanced up to 10 minutes. Meanwhile the seed germination percentage was reduced to 34 - 65 per cent. Bavistin although not very effective in terms of microbe elimination, it did not damage plant tissue as the chemical is easy to remove and is a relatively 'soft' sterilant, so use of only bavistin gave the least sterilization. While mercuric chloride is probably the most efficient sterilant, and $HgCl_2$ (0.1%) with 8-10 min duration gave the better results, but this was still not efficient as the residual of $HgCl_2$, specifically, mercury,

suppressed the germination of rice seeds. Similar results for efficiency of mercuric chloride as surface sterilizing agents for rice has been demonstrated in various experiments (Illahi *et al.*, 2005; Sikder *et al.*, 2006). There were clear exudates at the interface between the medium and the germinating seeds. However, no presence microbial growth was detected in them when these exudates were kept for longer duration. The exuded substances may be a long chain polysaccharide as some species are known to release large amounts of these into the medium after several weeks in culture. In our study, best results were obtained using bavistin 1.0% + $HgCl_2$ 0.1% in combination for 6 min with a germination percentage of 70 to 80%. This sterilization regime was therefore selected in subsequent experimentations as it is quite simple.

Table 2. Effect of different seed surface sterilants and treatment duration on seed germination in two rice varieties

Varieties/ Treatment Duration (min.)	PAU 201	PR 116	Mean	PAU 201	PR 116	Mean
	Germination (%)			Contamination (%)		
Mercuric chloride (0.10%)						
4	73.45 (58.98)	67.75 (55.39)		9.62 (18.06)	7.48 (15.67)	
6	77.70 (61.80)	75.55 (60.36)		6.66 (14.95)	5.28 (18.26)	
8	61.58 51.69)	59.37 (50.40)		5.51 (13.57)	3.16 (10.23)	
10	43.50 (41.26)	37.67 (37.80)		3.59 (10.92)	2.12 (8.37)	
Bavistin (1.00%)						
4	62.25 (52.09)	66.64 (54.71)		12.37 (20.59)	10.01 (18.44)	
6	75.65 (60.43)	78.42 (62.31)		12.07 (20.32)	7.66 (16.06)	
8	71.00 (57.41)	77.32 (61.56)		7.50 (15.89)	5.08 (13.02)	
10	59.70 (50.59)	64.85 (53.63)		5.01 (12.93)	4.36 (12.05)	
Bavistin (1.00%) + Mercuric chloride (0.10%)						
4	68.62 (55.96)	71.42 (57.68)		4.54 (12.30)	6.12 (14.32)	
6	78.42 62.31)	80.00 (63.43)		1.00 (5.73)	1.90 (7.92)	
8	51.47 (45.84)	61.62 (51.71)		0.00 (0.00)	1.25 (6.41)	
10	31.50 (34.14)	33.45 (35.33)		0.00 (0.00)	0.00 (0.00)	

CD at (.05) Genotypes (G) 0.13; Sterilants (S) 0.11 ; G x S 0.23; Durations (D) 0.13 ; G x D 0.27; S x D 0.23 ; G x S x D 0.47

The values in parantheses are arcsin transformed values

CONCLUSIONS

We studied the effects of different media and sterilization methods on seed germination of two rice varieties *in vitro* to produce aseptic seedlings for tissue culture and genetic transformation studies. Out of the two basal media formulations tested for seed germination, full strength MS salts and vitamins supported the higher percentage of seed germination (76.59%) than half-strength MS salts and vitamins (73.32%). Among the sterilization methods studied, the percentage of seed germination and contamination varied with the sterilizing agent and duration of treatment. The sterilization agents were arranged as per their effectivenss are: mercuric chloride (0.1%) + bavistin (1.0%), mercuric chloride (0.1%), and bavistin (1.0%) based on minimum contamination and high germination. Of the various sterilant and duration combinations tested, Bavistin 1.0% + $HgCl_2$ 0.1% gave the best surface disinfection with minimum contamination of seed and germination as good as (75 to 80%) when treatment duration was 6 minutes in both the rice cultivars, so it is recommended for production of axenic seedlings *in vitro* to collect aseptic explants which is a prerequisite for *in vitro* regeneration.

REFERENCES

Afolabi A.S., Oyebanji, O., Odusanya, M.E., Abo, M., Misra and Ogbadu G.H. 2008. Regeneration of plants from rice caryopsis derived callus culture of Nigerian local cv. Suakoko 8 and a NERICA cv. FARO 55. *African Journal of Plant Science*, **2**:109-112.

Al-Khayri, J., C. Shamblin, R. McNew and E. Anderson. 2001. Callus induction and plant regeneration of U.S. rice genotypes as affected by medium constituents. *In vitro Cellular and Developmental Biology-Plant*, **32**: 227-232.

Baker, R and Phillips, D.J. 1962. Obtaining pathogen free stock by shoot tip culture. *Phytopathology*, **63**: 1242-244.

Chun, S.C. and Schneider, R.W. 1993. The effect of pH of household bleach solutions used as a surface sterilant for rice seed. (Abstr.) *Phytopathology*, **84**:775.

Gamborg, O.L. and Phillips, G.C. 1995. Sterile Techniques.p. 35-42. In: *Plant Cell, Tissue and Organ Culture, Fundamental Methods* (O. L. Gamborg and G. C. Phillips, eds.). Springer-Verlag Berlin, Heidelberg, New York.

Gamborg, O.L., Miller, R. and Ojima, K. 1968. Nutrient requirements of suspension cultures of soybean root cells. *Experimental Cell Research*, **50**:151–58.

George, E.F. 1993. *Plant Propagation by Tissue Culture*, Part 1, The Technology, Equipment and procedures, pp.95-130, Exegetics Ltd., Edington, Wilts., England.

Hoque, M.N., R. Lutfur. and H. Lutful. 2007. Effect of culture media on seed dormancy and callus induction ability of some wild and cultivated rice genotypes. *Biotechnology*, **6**: 61-63.

Ikram-ul-Haq., Dahri, A.M., Dahot, M.U., Parveen, N., Ghaffar, A. and Laghari, A.L. 2010. Growth responses of NaCl stressed rice (*Oryza sativa* L.) plants germinated

from seed in aseptic nutrient cultures supplemented with praline. *Afr. J Biotechnol.*, 9: 6534-6538.

Ilahi, I., Bano, S., Jabeen, M. and Rahim, F. 2005. Micropropagation of rice (*oryza sativa* l. Cv Swat-ii) through somatic embryogenesis. *Pak. J. Bot.*, **37**: 237-242.

Kant, P., Kant, S., Jain, R.K. and Chaudhury, V.K. 2007. *Agrobacterium* mediated high frequency transformation in dwarf recalcitrant rice cultivars. *Biologia Plantarum*, **5**:61-68.

Karthikeyan, A., Pandian, S.T.K., and Ramesh M. 2008. High frequency plant regeneration from embryogenic callus of a popular *Indica* rice (*Oryza sativa* L.). *Physiology and Molecular Biology of Plants*, **15**: 371-375.

Kreider, J.W. 1968. Some practical aspects of the use of laminar air flow systems for tissue culture manipulations. *Appl. Microbiol.*, **16**: 1804-805.

Lindsey, D.L. 1967. Growth of beans, tomatoes and corn under gnotobiotic conditions. *Phytopathology*, **63**:1501-504.

Mariani, T.S., Miyake, H. and Takeoka, Y. 2003. Improvement of direct somatic embryogenesis in rice by selecting the optimal development stage of explant and applying desiccation treatment. *Plant Production Science*, **3**: 114-123.

Meneses, A., Flores, D., Muñoz, M., Arrieta, G. and Espinoza, A.M. 2005. Effect of 2,4-D, hydric stress and light on *indica* rice (*Oryza sativa*) somatic embryogenesis. *Int. Biol. Trop.*, **53**: 361-368.

Miflin, B.J. 1969. A technique for the sterile culture of germinating barley embryos. *J. Exp. Bot.*, **20**: 805-809.

Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bio-assays with tobacco tissue cultures. *Physiologia Plantarum*, **15**: 508-09.

Rahman A.R., Roowi, S., Zaliha, W. and Subramaniam, S. 2010. Regeneration of Malaysian Indica Rice (*oryza sativa*) Variety Mr232 via optimised somatic Embryogenesis System. *Journal of Phytology*, **2**: 31-36.

Shahsavari, E., Maheran, A.A., Akmar, A.S.N. and Hanafi, M.M. 2010. The effect of plant growth regulators on optimization of btissue culture system in Malaysian upland rice. *Afr. J. Biotechnol.*, 9: 2089-2094.

Sikder, M.B.H., Sen, P.K., Abdullah-Al, M., Ali, M.R. and Rahman, S.M. 2006. *In Vitro* Regeneration of Aromatic Rice *(Oryza sativa* L.). *Afr. J. Biotehnol.*, 8: 759-762.

Summart, J., Panichajakul, S., Prathepha, P. and Thanonkeo, P. 2008. Callus induction and influence of culture condition and culture medium on growth of thai aromatic rice, Khao Dawk Mali 105, cell culture. *World Applied Sciences Journal*, **5**: 246-251.

Wenzhong, T., Rance, I., Sivamani, E. 1994. Improvement of plant regeneration frequency *in vitro* in *indica* rice. *Chin. J. Genet.*, **21**:1-9.

White, P.R. 1943. Germ free plants and plant parts as material for physiological and pathological studies. p. 188-204. In: *Micrurgical and Germ Free Techniques: Their Application to Experimental Biology and Medicine* (Reyniers J. A., ed).