INFLUENCE OF POTASSIUM DIHYDROGEN PHOSPHATE ON CALLUS INDUCTION AND PLANT REGENERATION IN RICE (*ORYZA SATIVA* L.)

MRANALI CHAUHAN AND S.L. KOTHARI*
Department of Botany, University of Rajasthan, Jaipur, India 302004

ABSTRACT

The effect of potassium dihydrogen phosphate (KH_2PO_4) on callus induction and plant regeneration from callus cultures of indica rice (*Oryza sativa* cultivar Pusa Basmati-1) was investigated. Callus was induced on Murashige and Skoog (MS) medium supplemented with 11.31 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and subsequent plant regeneration was obtained on MS medium containing 2.68 μM α-naphthaleneacetic acid (NAA) and 8.87 μM 6-benzylaminopurine (BAP). Both the induction and the regeneration media were supplemented with different levels of KH_2PO_4 (0 to12.50 mM). The level of KH_2PO_4 in the induction medium influenced the percent water content of the callus. KH_2PO_4 was found to be essential for plant regeneration as no shoot regeneration occurred in the absence of KH_2PO_4. Highest number of shoots per explant was obtained when KH_2PO_4 level in the callus induction and plant regeneration medium was 0.625 mM (half of MS level) and 1.25 mM (normal MS level) respectively. By making amendments and modifications of the KH_2PO_4 level in the induction medium, the regeneration increased 2.5 folds. Almost similar regeneration frequency was observed from callus induced on lower levels of KH_2PO_4. Regenerated shoots were rooted on MS medium supplemented with 2.68 μM NAA. Rooted plantlets were transferred to soil where they survived and set seeds.

Key-words: potassium dihydrogen phosphate, callus induction, plant regeneration, water content, indica rice

*Corresponding author: Ph./Fax +91(141)2703439; e-mail: SLKothari@Lycos.com

DOI: 10.1556/CRC.33.2005.2-3.119

INTRODUCTION

Rice is one of the most important staple crops in the world and indica rice varieties are the principal food source in most of the world's tropical regions. Since the first report of plant regeneration from rice callus (Nishi et al. 1968), successful tissue culture has been reported from various explants including mature and immature embryos (Koetje et al. 1989; Grimes and Hodges 1990; Lee et al. 2002); proembryos (Zhao et al. 1999); leaf sheath (Abdullah et al. 1986); leaf bases (Wernicke et al. 1981); root-segments (Sticklen 1991); young inflorescences (Chen et al. 1985); coleoptile (Oinam and Kothari 1995). The response in terms of absolute number of plants regenerated per explant is poor in important indica cultivars. It has been suggested that these efficiencies could be increased if appropriate amendments are made to the tissue culture medium. Researches in the recent years have shown that different cereals require different composition of the basal medium depending upon the genotype, source and age of the explant, plant growth regulators (PGRs), and culture conditions applied. The composition of the culture medium is considered important, as tissue culture responses are highly dependent on the interplay of mineral nutrient concentrations with other factors (Leifert et al. 1995; Preece 1995; Ramage and Williams 2002). An optimized concentration of nutrients can even partially substitute for PGRs (Preece 1995; Poddar et al. 1997). Therefore, the composition of the basal medium has been routinely modified for different cereal crops (Grimes and Hodges 1990; Purnhauser 1993; Sahrawat and Chand 1999; Dahleen and Bregitzer 2002; Nirwan and Kothari 2003). Higher concentration of macro-nutrients enhances the frequency and development of embryogenic callus in wheat (He et al. 1989). In indica rice also improved plant regeneration has been achieved by optimizing the concentration of nutrient salts (Raval and Chattoo 1993; Khanna and Raina 1997, 1978; Sahrawat and Chand 1999, 2002). The present investigation was undertaken to optimize the KH_2PO_4 level in the basal medium for efficient callus induction and high plant regeneration of indica rice to facilitate the regeneration of high number of plants after genetic transformation.

MATERIALS AND METHODS

Mature seeds of indica rice (*Oryza sativa* L. cultivar Pusa Basmati-1) were used for embryogenic callus induction and plant regeneration. The seeds were procured from GB Pant University of Agriculture and Technology, Pant Nagar, Uttaranchal. Mature seeds were dehusked, surface sterilized with 0.1% aqueous solution of mercuric chloride for 8 minutes followed by three rinses with sterile distilled water. The basal MS medium (Murashige and Skoog, 1962) supplemented with 11.31 µM 2,4-dichlorophenoxyacetic acid (2,4-D), different concentrations of potassium dihydrogen phosphate (KH_2PO_4; 0 to 12.50 mM) and sucrose (3.0%, w/v) was used for callus induction. The pH of the medium was adjusted to 5.8 before being solidified

with 0.8% bacteriological grade agar (Qualigens, India) and autoclaved at 121°C and 1.06 kg/cm^2 pressure for 15 minutes. The cultures were maintained at 26±1°C under a photoperiod of 16/8 hrs (day/night) and at a light intensity of 25 μmol $m^{-2}s^{-1}$.

Plant regeneration: The calli were transferred on regeneration medium after 4 weeks without exerting any visual selection. From each treatment half the number of embryo-derived calli were transferred to regeneration medium consisting of MS medium with KH_2PO_4 concentration as per the induction medium (R_M medium) and the other half were transferred to the MS medium without any modification of basal salts (R_N medium). Both the regeneration media R_M and R_N were supplemented with a combination of 2.68 μM α-naphthaleneacetic acid (NAA) and 8.87 μM 6-benzylaminopurine (BAP). Regenerated shoots over 0.5 cm in height were counted after 4 weeks of transfer. Well-developed shoots (over 1 cm) were transferred on rooting medium (0.2% w/v phytagel solidified MS containing 2.68 μM NAA). Plantlets with good root system were transferred to soil.

The data were recorded on percent water content of the callus after four weeks of incubation on induction medium. The percent water content was determined from the difference between the fresh weight and the dry weight, divided by the fresh weight. The dry weight was obtained by drying the callus in an oven at 60°C for 24 hrs.

Statistical analysis- Data for percent water content were analysed using a one way analysis of variance (ANOVA) and comparisons between the mean values of treatments were made by the least significantly difference (LSD) test.

RESULTS AND DISCUSSION

Seeds inoculated on MS medium supplemented with 2,4-D and various levels of KH_2PO_4 produced creamish compact embryogenic type of callus along with friable non-embryogenic callus. The percent water content varied with the concentration of KH_2PO_4 in the medium (Table 1). As the concentration of KH_2PO_4 was raised, the water content of the callus increased. Calli induced on lower concentration of KH_2PO_4 were relatively dry and compact in appearance. These calli gave rise to larger number of shoots on R_N regeneration medium as compared to those induced on higher concentrations and looked watery. Reduced water content is known to increase embryogenesis and plant regeneration in rice (Rance et al. 1994; Jain et al. 1996). In the present study, change in the percent water content of the callus was observed by changing the concentration of KH_2PO_4 in the callus induction medium.

Not only the callusing response but also the regeneration was dependent on the concentration of KH_2PO_4 in the medium. Plants could not be regenerated on medium devoid of KH_2PO_4. The regeneration percentage, average plants/regenerating embryo

Table 1. Water content of seed derived callus induced on MS medium supplemented with 2,4-D (11.31 μM) and different levels of KH_2PO_4

Conc. of KH_2PO_4 in callus induction medium	Percent water content* of the callus
0	81.66±3.29 a
0.625 mM	86.17±0.46 b
1.25 mM	86.58±1.73 b
2.50 mM	87.24±1.35 bc
3.75 mM	87.90±1.35 bc
5.0 mM	88.62±1.50 c
6.25 mM	89.10±0.53 c
12.50 mM	91.64±1.06 d

* Percent water content = [(Fresh weight - Dry weight) / Fresh weight] X 100
Values followed by the same alphabet are not significantly different from each other (P=0.05)

Table 2. Regeneration response of callus derived from seeds of *Oryza sativa* cultivar Pusa Basmati-1.

Conc. of KH_2PO_4 in callus induction* medium	Regeneration medium	Percentage regeneration	No. of plants per regenerating seed Mean±SD	Total plantlets Seeds=50
0	R_N	80	3.50±1.0	140
0.625 mM		80	40.50±5.65	1620
1.25 mM		80	16.75±7.32	670
2.50 mM		75	7.33±4.93	275
3.75 mM		50	6.50±2.12	163
5.0 mM		47	3.0±1.414	100
6.25 mM		0	-	-
12.50 mM		0	-	-
0	R_M	0	-	-
0.625 mM		60	3.33±1.52	105
1.25 mM		80	16.75±7.32	670
2.50 mM		60	7.0±3.60	210
3.75 mM		60	6.66±4.04	200
5.00 mM		50	4.0±0	100
6.25 mM		40	4.5±3.53	90
12.50 mM		33	3.0±0	50

* MS medium + 2,4-D (11.31 μM)
SD: Standard Deviation
R_N: MS medium + NAA (2.68 μM) + BAP (8.87 μM) + KH_2PO_4 (1.25 mM)
R_M: MS medium + NAA (2.68 μM) + BAP (8.87 μM) + KH_2PO_4 level as in callus induction medium

and total plantlets obtained from fixed number of seeds for each treatment is given in Table 2. While calculating average plants/seed, only the regenerating seeds were taken into account. This value correlates better with the visual observation of regeneration. Total plantlets were counted on the basis of fixed sample size (50 seeds) whether regenerating or non-regenerating. Variation in the response between treatments is clearly observed when total number of plantlets was compared.

The regeneration response of calli was better on R_N medium, indicating that the level of KH_2PO_4 in the MS though optimum for regeneration of plants but is not suitable for efficient induction of embryogenic calli. The calli induced on various concentrations of KH_2PO_4 had different efficiencies to regenerate plants even on the same regeneration medium. The inorganic constitution of the callusing medium affected the regeneration response. Of the various concentrations evaluated at the induction, 0.625 mM gave the most clear-cut effect. The percentage of regenerating calli remained unchanged when the concentration of KH_2PO_4 was reduced in the medium but the total number of regenerated plants changed considerably. The callus induced even in the absence of KH_2PO_4 showed percentage of regeneration comparable to the control i.e. 80%. At 0.625 mM the percentage regeneration still remained 80% but the total number of regenerated plantlets was highly increased. At higher levels of KH_2PO_4 both the percentage of responding cultures as well as the total number of regenerated plantlets was reduced. By making amendments and modifications of the KH_2PO_4 level in the induction medium, the regeneration has been increased by 2.5 folds from an average of 670 plants regenerated from 50 seed explant in initial experiment to an average of 1620 plants after optimization. The regenerated plantlets had normal roots and successfully survived in the field.

The results of the present study showed that different levels of KH_2PO_4 are required at the induction and regeneration phases. While 0.625 mM KH_2PO_4 was beneficial for callus induction, optimum regeneration occurred on 1.25 mM of KH_2PO_4. A differential requirement of the composition of basal medium at the two phases of development has been previously reported by Khanna and Raina 1998; Kothari et al. 2004.

In wheat, barley, rice and sorghum, optimization of copper sulphate level increased the number of green plants (Purnhauser 1991; Dahleen 1995; Sahrawat and Chand 1999 and Nirwan and Kothari 2003). A significant enhancement of plant regeneration has been achieved by increased levels of ammonium nitrate, potassium nitrate, ammonium sulphate in rice (Khanna and Raina 1997; Sahrawat and Chand 2002) and *Eleusine* (Poddar et al. 1997). This is the first report to investigate the effect of KH_2PO_4 on callus induction and plant regeneration in indica rice. The exact mechanism for the enhancement of regeneration is not known, however the two nutrients - potassium and phosphate are implicated in the osmotic adjustment and energy metabolism of the cell respectively. Higher concentration of KH_2PO_4 can also limit the availability of other mineral ions such as Fe^{3+} and Ca^{2+} (Loneragan and Asher 1982).

The present study concludes that the level of KH_2PO_4 in the MS medium is not optimal for rice tissue culture. Furthermore, the requirement for the induction and regeneration phases is not the same. Differential optimization of the two phases resulted in an efficient callus induction and high frequency plant regeneration in indica rice.

ACKNOWLEDGEMENT

Research fellowship to MC provided by Council of Scientific and Industrial Research (CSIR), HRDG, New Delhi (India) is gratefully acknowledged.

REFERENCES

Abdullah R, Cocking EC, Thompson JA (1986) Efficient plant regeneration from rice protoplasts through somatic embryogenesis. Bio/Technol 4:1087-1090

Chen TH, Lam L, Chen SH (1985) Somatic embryogenesis and plant regeneration from cultured young inflorescences of *Oryza sativa* L. (rice). Plant Cell Tiss Org Cult 4:51-54

Dahleen LS (1995) Improved plant regeneration from barley callus cultures by improved copper levels. Plant Cell Tiss Organ Cult 43:267-269

Dahleen LS, Bregitzer P (2002) An improved media system for high regeneration rates from barley immature embryo-derived callus cultures of commercial cultivars. Crop Sci 42:934-938

Grimes HD, Hodges TK (1990) The inorganic NO_3^-: NH_4^+ ratio influences plant regeneration and auxin sensitivity in primary callus derived from immature embryos of indica rice (*Oryza sativa* L.). J Plant Physiol 136:362-367

He DG, Yang YM, Scott KJ (1989) The effect of macronutrients in the induction of embryogenic callus from immature embryos of wheat (*Triticum aestivum* L.). Plant Sci 64:251-258

Jain RK, Jain S, Wu R (1996) Stimulatory effect of water stress on plant regeneration of aromatic indica rice varieties. Plant Cell Rep 15:1449-1459

Khanna HK, Raina SK (1997) Enhanced plant regeneration in basmati rice (*Oryza sativa* L. cv. Karnal Local) embryo calli through modification of NO_3^- + $NH4^+$ concentrations. J Biochem Biotech 6:75-80

Khanna HK, Raina SK (1998) Genotype X culture media interaction effects on regeneration response of three indica rice cultivars. Plant Cell Tiss Org Cult 52:145-153

Koetje DS, Grimes HD, Wang YC, Hodges TK (1989) Regeneration of indica rice (*Oryza sativa* L.) from primary callus derived from immature embryos. J Plant Physiol 135:184-190

Kothari SL, Agarwal K, Kumar S (2004) Inorganic nutrient manipulation for highly improved *in vitro* plant regeneration in finger millet - *Eleusine coracana* (L.) Gaertn. In Vitro Cell Dev Biol (in press)

Lee K, Jeon H, Kim M (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 Tiss Org Cult 17:237-244

Leifert C, Murphy KP, Lumsden PJ (1995) Mineral and carbohydrate nutrition of plant cell and tissue cultures. Crit Rev Plant Sci 14(2):83-109

Loneragan JF, Asher CJ (1982) Response of plants to phosphate concentration in solution culture: II. Rate of phosphate absorption and its relation to growth. Soil Sci 103:311-318.

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

Nirwan RS, Kothari SL (2003) High copper levels improve callus induction and plant regeneration in *Sorghum bicolor* (L.) Moench. In Vitro Cell Dev Bio 2:165-168

Nishi T, Yamada Y, Takahashi E (1968) Organ differentiation and plant restoration in rice callus. Nature 219:508-509

Oinam GS, Kothari SL (1995) Totipotency of coleoptile tissue in indica rice (*Oryza sativa* L. cv. CH 1039). Plant Cell Rep 14:245-248

Poddar K, Vishoni RK, Kothari SL (1997) Plant regeneration from embryogenic callus of finger millet *Eieusine coracana* (L.) Gaertn. on high concentration of NH_4NO_3 as a replacement of NAA in the medium. Plant Sci 129:101-106

Preece JE (1995) Can nutrient salts partially substitute for plant growth regulators? Plant Tiss Cult Biotech 1:26-37

Purnhauser L (1991) Stimulation of shoot and root regeneration in wheat *Triticum aestivum* callus cultures by copper. Cereal Res Comm 19:419-423

Purnhauser L, Gyulia G (1993) Effect of copper on shoot and root regeneration in wheat, triticale, rape and tobacco tissue cultures. Plant Cell Tiss Organ Cult 35:131-139

Ramage CM, Williams RR (2002) Mineral nutrition and plant morphogenesis. In Vitro Cell Dev Biol 38:116-124

Rance IM, Tian W, Mathews H, Kochko A, Beachy RN, Fauquet C (1994) Partial desiccation of mature embryo-derived calli, a simple treatment that dramatically enhances the regeneration ability of indica rice. Plant Cell Rep 13:647-651

Raval M, Chattoo BB (1993) Role of media constituents and proline in callus growth, somatic embryogenesis and regeneration of *Oryza sativa* cv indica. Ind J Exp Bio 31:600-603

Sahrawat AK, Chand S (2002) Influence of ammonium nitrate on plant regeneration in indica rice (*Oryza sativa* Linn.). Ind J Biotech 1:367-371

Sahrawat AK, Chand S (1999) Stimulatory effect of copper on plant regeneration in indica rice. J Plant Physiol 154:517-522

Sticklen MB (1991) Direct somatic embryogenesis and fertile plants from rice root cultures. J Plant Physiol 138:577-580

Wernicke W, Brettel R, Wakizuma T, Potrykus I (1981) Adventitious embryoid and root formation from rice leaves. Z Planzenphysiol 103:361-365

Zhao J, Zhou C, Yang HY (1999) *In vitro* development of early proembryos and plant regeneration via microculture in *Oryza sativa*. Plant Cell Tiss Org Cult 55:167-174

Received 18 June, 2004, accepted 8 February, 2005