ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 5 (3): 379-384, 2006
ISSN 1682-296X

Stimulatory Effects of Casein Hydrolysate and Proline in *in vitro* Callus Induction and Plant Regeneration from Five Deepwater Rice (*Oryza sativa* L.)

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Abstract: Interactive effects of genotypes with callus induction and plant regeneration medium combinations on callus induction and plantlet regeneration response were studied for five deepwater Indica rice cultivars namely; Habiganj Aman-1, Habiganj Aman-2, Habiganj Aman-8, Murabajal and Gheoch. Mature seed scutellums were cultured on MS and LS basal media supplemented with different combinations of 2,4-D, casein hydrolysate (CH) and proline. In cv. HA-8, basal medium combination of MS+2 mg L^{-1} 2,4-D supplemented with 0.6% (w/v) CH was found to be the best for callogenesis where callusing frequency was 87%. The growth rate of callus was frequently increased by the addition of different concentrations of CH with callus induction media. On the other hand, when proline was supplemented in to callus induction media it had no residual effect on callus growth. Embryogenic calli were transferred on MS and LS based regeneration media supplemented with 2 mg L^{-1} BAP and also different concentrations of casein hydrolysate and proline. The highest regeneration frequency (80%) was observed in cv. HA-1 on LS basal media supplemented with only 2 mg L^{-1} BAP. However, present study demonstrated that plant regeneration media supplemented with proline is not inhibitory for plant regeneration but have a noticeable comparatively stimulating effect on regeneration from callus. Here combination of casein hydrolysate tremendously reduced plant regeneration. An over all analysis of variations of frequencies for callusing and plant regeneration revealed a contrasting interaction among the culture media and genotypes.

Key words: Deepwater rice, callus, regeneration, casein hydrolysate, proline, medium interaction

INTRODUCTION

From the pre historic era to the modern times, rice has been the most important source of human nutritions and has help sustain the increasing population and the development of human civilization. Rapid and remarkable recent advances in genetic engineering could result in genetically modified rice plants producing completely novel products. Rice would be the ideal for this purpose, especially deepwater rice sustains millions of subsistence farmer in South and Southeast Asia. It is endemic to Bangladesh. *In vitro* techniques for culture of the plant cells, tissues and organs have advanced dramatically during the last few decades due to the great advantage of plant biotechnology. The use of meristematic tissue such as immature and mature embryos, young inflorescences and the base of mature leaves at a defined stage of development provide the basis for most rice tissue culture systems. Plant regeneration from the major cultivated Indica rice varieties is generally poor (Abe and Futsuhara, 1984; Kavi Kishor and Reddy, 1986). An important trait of plants derived from somatic embryos, in their uniformity and genetic stability (Vasil, 1995). The different combinations of medium may influence the variations of callus formation and plant regeneration. In this investigation effect of supplementation of callus induction and plant regeneration media with Casein Hydrolysate (CH) and proline has been studied. An observation by Murashige and Skoog (1962) that the presence of CH allowed vigorous organ development over broader range of IAA and Kinetin level. Proline is one kind of amino acid. Amino acids provides plant cells with an immediately available source of nitrogen and uptake can be much more rapid than an organic constituent in the same medium (Thom, 1981). It has been reported that the enhancing effect of plant regeneration by a dehydration treatment of rice callus coincides with the decline of the proline content of the callus. Reproducible and efficient protocol of rice through somatic embryogenesis from callus has been developed (Al-Forkan *et al*., 2005). The number, mass and morphology of the callus formed on the scutellum were dependent on the medium tested. National and International efforts have been made in the past for improving deepwater rice cultures. However, keeping in

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the mind, the magnitude of the problem related to cultivation of deepwater rice and continuing suffering of millions of farmers in Bangladesh, the necessity of research to develop cell to plant system in the deepwater rice has been given priority. The successful application of available gene transfer technology to rice will only be possible when reproducible regeneration systems are routinely available. In this study reproducible and efficient plant regeneration system has been established.

MATERIALS AND METHODS

The experiment was conducted at Plant Tissue Culture Laboratory in the Department of Botany, University of Chittagong, Bangladesh during 2004-2005. Five deepwater rice (*O. sativa* L.) cultivars namely; Habiganj Aman-1, Habiganj Aman-2, Habiganj Aman-8, Murabajal and Gheoch were used in this study. The MS (Murashige and Skoog, 1962) and LS (Linsmaier and Skoog, 1965) based basal media with different hormonals and additives combinations with 30 g L^{-1} sucrose and 0.8% (w/v) agar solidified media were used for enhancing callus from mature seed scutellum of selected cultivars. MS and LS basal media were supplemented with different combinations of 2 mg L^{-1} 2,4-D; 2 mg L^{-1} 2, 4-D+0.25 mg L^{-1} proline+0.1% (w/v) CH; 2 mg L^{-1} 2, 4-D+0.2% (w/v) CH, 2 mg L^{-1} 2,4-D+0.4% (w/v) CH and 2 mg L^{-1} 2,4-D+0.6% (w/v) CH. MS and LS based plant regeneration media were also supplemented with different combinations and concentrations of plant growth regulators and additives such as 2 mg L^{-1} BAP (RM-1, RM-4); 2 mg L^{-1} BAP+0.25 mg L^{-1} 2,4-D+0.25 mg L^{-1} proline (RM-2, RM-5) and 2 mg L^{-1} BAP+0.1% (w/v) CH (RM-3, RM-6) were used for this purpose. In the parenthesis RM-1, RM-2 and RM-3 referred as MS based and later RM-4, RM-5 and RM-6 referred as LS based media, respectively.

Seeds were dehusked manually and sterilized by soaking seeds in 0.2% (w/v) $HgCl_2$ for 15 min and washed thoroughly (5-6 times) with sterile glass-distilled water. Then, seeds were placed in petri dish contained 20 ml callus induction medium and incubated at 26±2°C in dark. After 14 day the number of calli induced (from MSS) per petri-dish was counted, recorded and elongated shoots and roots were removed. Then seeds attached with scutella derived callus were cultured freshly on respective medium for another 14 day under the same growth condition as mentioned before. MSS derived embryogenic calli were transferred to regeneration medium where media were semi solidified with 1% (w/v) agar and incubated in the dark at 26±1°C for 10 days. After that calli were sub-cultured on the same medium except medium was semi-solidified with 0.8% (w/v) agar and kept under a 16 h photoperiod at 26±1°C for three weeks. The shoot regeneration frequencies were recorded 20 day after transfer of tissues to regeneration medium at the percentage of scutella derived calli each producing one or more shoots. Each shoot (2-3 cm in height) was detached from individual calli and each shoot was multiplied by transferring for 20-30 day on regeneration medium. After that regenerates were cultured on MS+1.5 mg L^{-1} NAA rooting medium. Well-developed plantlets were then transferred to the natural condition in pot.

RESULTS

Two types of calli formed at the scutella region of the cultured seeds. One type of callus was compact and nodular in structure and called embryogenic callus (Fig. 1a). The other type of callus was friable, translucent and slimy which never formed embryoids and called non-embryogenic callus. However, this type of callus was proliferated faster. The highest percentage of callus production 87 and 82% were obtained on MS and LS based media, respectively, where both the basal media supplemented with 2 mg L^{-1} 2,4-D+0.6% (w/v) CH in cv. HA-8 (Table 1). Significant differences on percentage of callus production were recorded on among the media such as 2 mg L^{-1} 2,4-D; 2 mg L^{-1} 2,4-D+0.25 mg L^{-1} proline+0.1% (w/v) CH and 2 mg L^{-1} 2,4-D with 2-6% (w/v) CH. In general, calli from all the cultivars were

Table 1: Comparison of callusing percentage on MS or LS based media supplemented with different combinations of plant growth regulators and additives

	% of callus initiation									
	MS or LS+2 mg L^{-1} 2,4-D		MS or LS+2 mg L^{-1} 2,4-D+0.25 mg L^{-1} proline+0.1% CH		MS or LS+2 mg L^{-1} 2,4-D supplemented with					
					0.2% CH		0.4% CH		0.6% CH	
Cultivars	MS	LS	MS	LS	MS	LS	MS	LS	MS	LS
HA-1	36	33	48	40	55	50	79	77	85	80
HA-2	38	36	41	38	45	40	48	44	58	51
HA-8	49	42	63	56	63	53	81	78	87	82
Murabajal	58	53	44	38	65	60	74	72	76	73
Gheoch	24	20	45	40	45	35	64	78	67	82

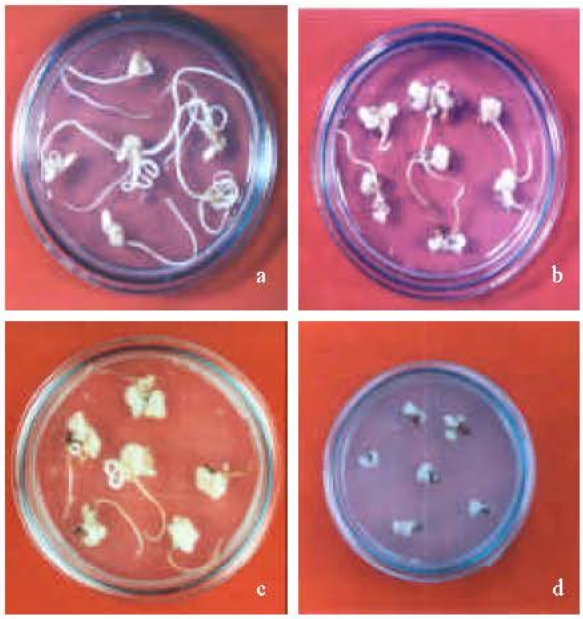

Fig. 1(a-d): Effect of supplementation of callus induction medium with different concentration of casein hydrolysate on nature of callus at 14 days (a) embryogenic callus induced on 0.2% (w/v) casein hydrolysate (CH) supplemented callus induction medium b) embryogenic callus induced on 0.4% (w/v) CH medium (c) embryogenic callus induced on 0.6% (w/v) CH medium (d) callus induced from MSS on MS based CH free medium

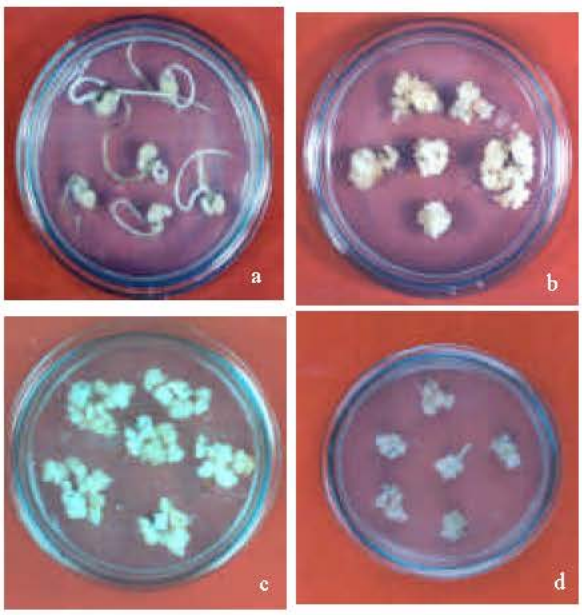

Fig. 2 (a-d): Effect of addition of casein hydrolysate and proline in callus induction medium at 28 days (a) callus induced from MSS on MS based callus induction medium with 0.2% (w/v) CH (b) MSS-derived callus on the same medium with 0.4% (w/v) CH (c) MSS-derived callus on the same medium with 0.6% (w/v) CH (d) callus on MS based medium supplemented with 0.25 mg L^{-1} proline

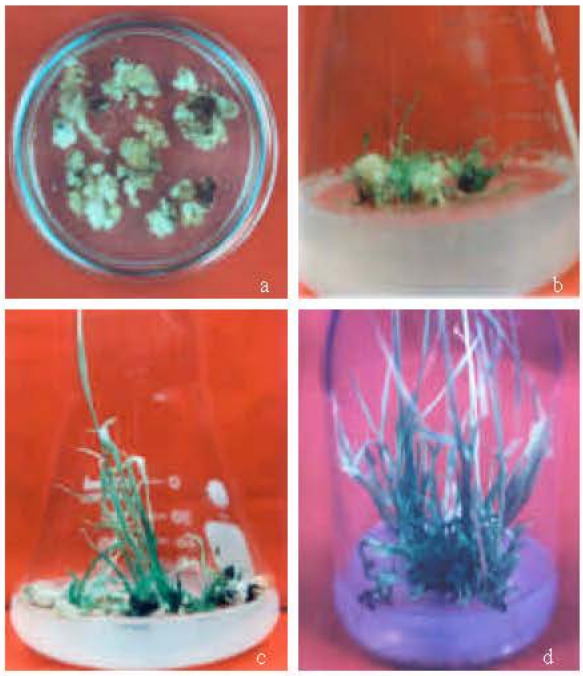

Fig. 3 (a-d): Effect of supplementation of plant regeneration medium with casein hydrolysate and proline on plant regeneration (a) callus showing yellowish embryogenic nature upon transferred to LS based regeneration medium supplemented with 2.0 mg L^{-1} BAP+0.1% (w/v) CH+1% (w/v) agar (b) Differentiation of shoot/buds from MSS-derived callus cultured on MS-based proline supplemented medium after 5 days at light conditions (c) Addition of CH in the regeneration medium showing reduced plant regeneration (d) Addition of proline in the regeneration medium showing enhanced plant regeneration

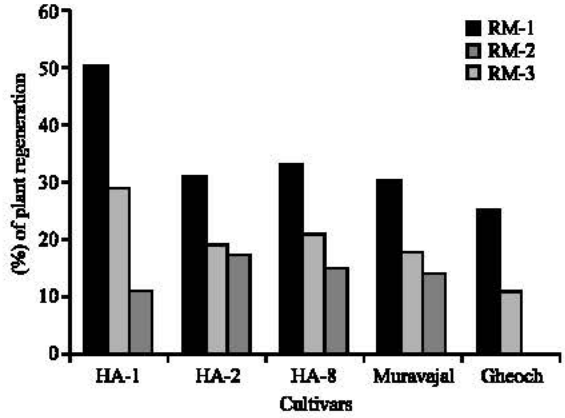

Fig. 4: Effects of casein hydrolysate and proline supplemented with MS medium on plant regeneration

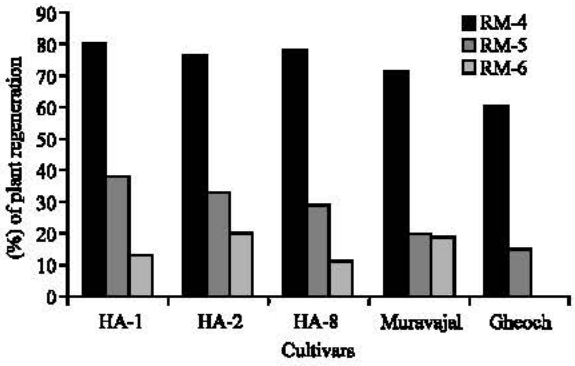

Fig. 5: Effects of casein hydrolysate and proline supplemented with LS medium on plant regeneration

compact, yellowish and big in size, well proliferated, some times dry, rooty and hardy. The supplementation of callus induction medium with CH tremendously increased callus production (Fig. 1a-c and 2a-c). In casein hydrolysate free MS and LS based media, which consisted only 2 mg L^{-1} 2,4-D (Fig. 1d), the frequency of calli formation were 36 and 33% which were comparatively lower than on MS and LS based media which were supplemented with 0.6% (w/v) CH where the percentage of calli were 85 and 80% in cv. HA-1. When proline was supplemented into the callus induction medium it had no residual effect on callus growth. Cultivar HA-2, cultured on MS and LS media

supplemented with 2 mg L^{-1} 2,4-D+0.25 mg L^{-1} proline+0.1% (w/v) CH were produced the lowest percentage of calli compared to other concentrations of CH added media (Fig. 2d). However, all the cultivars responded better on MS based callus induction medium compared to LS based callus induction medium (Table 1). Cultured callus of cv. HA-1 produced the highest percentage (50%) of plant on MS based media supplemented with 2 mg L^{-1} BAP (RM-1, Fig. 4). On the other hand, cv. Gheoch produced 25% of plant on the same medium. In contrast of that cv. HA-1 produced the highest percentage (80%) of plants on LS based RM-4 medium (Fig. 5). Interestingly, cv. Gheoch did not response on MS and LS based RM-3 and RM-6 medium, where media were supplemented with 2 mg L^{-1} BAP+0.1% (w/v) CH (Fig. 4). These results revealed that all the cultivars responded poorly in terms of plant regeneration on CH added RM-3 and RM-6 media. These types of calli were yellowish and watery (Fig. 3a). The shoot developed in CH supplemented media occasionally became brown and finally died. The addition of CH with MS and LS based regeneration media, the plant regeneration frequency tremendously reduced in all the cultivars.

Proline has a noticeable comparatively stimulating effect on plant regeneration from callus. Proline added in MS and LS based RM-2 and RM-5 media, cv. HA-1 produced comparatively higher percentage of plants 29% and 38% (Fig. 3b), respectively, than the CH added RM-3 and RM-6 media. On LS based proline supplemented medium (RM-5), cv. HA-1 produced deep green healthy plants with many shoots (Fig. 3d) whilst on medium RM-6; the same cultivars produced plants with fewer shoots (Fig. 3c). The percentage of plant regeneration varied genotype to genotype as well as different combination of medium.

DISCUSSION

Earlier studies have shown that embryogenesis and shoot regeneration are genetically determined in rice (Abe and Futsuhara, 1985). The present study has confirmed that the highest callus formation and plant regeneration are probably influenced by interaction of media components. It has been reported that the variation in embryogenic callus formation could be influenced by many other factors including differences in the media composition, concentrations of endogenous growth regulators and also addition of spermidine, casein enzymatic hydrolysate, proline, ascorbic acid and activated charcoal (Minhas *et al.,* 1999). CH can be a source of calcium, several micronutrients, vitamins and most importantly a mixture of up to 18 amino acids. Several investigators have concluded that CH itself is more effective for plant cultures than the addition of the major amino acids, which it's provide. This has led to speculation that CH might contain some unknown growth-promoting factor (Inoue and Maeda, 1982), which promoted callus growth. For cv. Murabajal, a higher frequency of plant regeneration was obtained on LS based RM-5 medium compared to RM-6 medium. This result demonstrates that proline is more relevant additive than CH. Many studies suggest that proline function in the intracellular osmotic adjustment between cytoplasm and vacuoles (Bandurska, 1993; Solomon *et al.,* 1994) and intracellular structures (Van Rensburg *et al*., 1993), a free radical scavenger (Smirnoff and Cumbes, 1989) or a strong compound of carbon and nitrogen for rapid recovery from stress (Jager and Meyer, 1977). Plant regeneration was also influenced by many other factors including the composition of the basal medium and nature of plant growth regulators added in the regeneration medium. A high frequency of plant regeneration was obtained from cv. HA-1 on RM-4 medium, which contained only 2 mg L^{-1} BAP as opposed to RM-6 medium, which contained 2 mg L^{-1} BAP+0.1% (w/v) CH. This demonstrates that the concentration of BAP is a more relevant growth regulator than a combination of BAP and 0.1% CH. Xie *et al.* (1995) reported that plant regeneration capability was dependent on the callus that was affected by the growth regulator combinations used in callus induction medium. In prepared mixture of amino acids resembling those in CH, competitive inhibition between some of the constituents was often observed the percentage of CH decreased plant regeneration. In this investigation cv. Gheoch cultured on MS and LS based RM-3 and RM-6 media, which contained 2 mg L^{-1} BAP+0.1% (w/v) CH did not produce plantlets. Ansist and Northcote (1973) reported that, the brand of CH known as N-Z amine' TM can produce toxic substances if concentrated solutions are heated or solutions are frozen and thawed several times. CH is therefore added in the media for shoot cultures, was found to hault plant regeneration and enhanced callus induction. This finding is contradict with the observation of Sheeja *et al*., (2004) where callus induction was haulted but plant regeneration enhanced by addition of CH in the medium. The probable reason might be due to differences between monocotyledons and dicotyledons plant. By observing overall response, it can be concluded that proline is not inhibitory for plant regeneration but CH inhibited formation and proliferation of plant growth. Furthermore, the calli, which survived, regeneration process delayed by 1-2 week depending on the concentration of CH. However, these studies clearly demonstrate that the

frequency of callus formation and efficient plant regeneration are mainly influenced by the plant genotype including composition of the culture media and plant growth regulators and additives. Furthermore, this experiment clearly demonstrated that CH could be added with callus induction medium for vigorous callus formation and proline could be added with plant regeneration medium for efficient plant regeneration. The protocol described in this study is recommended for high frequency of plant regeneration from deepwater rice cultivars as well as for production of transgenic rice plants with desired traits.

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