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AN EFFICIENT REGENERATION SYSTEM THROUGH *IN VITRO* SOMATIC EMBRYOGENESIS OF BARLEY (*HORDEUM VULGARE* L.)

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Abstract

This study was carried out to improve an efficient protocol for *in vitro* callus induction and plant regeneration using Bangladeshi barley genotypes collected from BARI, Gazipur, Bangladesh. After sterilization embryos were separated carefully from mature seeds of six barley genotypes (BB-1, BB-2, BB-3, BB-4, BB-5 and BB-6) and cultured them in MS medium supplemented with various concentration and combination of PGRs for callus induction and regeneration. Out of six genotypes BB-6 showed highest (38.17%) callus induction in MS + 4.0 mg/l 2,4-D + 200 mg/l L-proline + 300 mg/l casein hydrolysate; whereas, BB-4 and BB-5 showed no callus induction in the same medium. For plant regeneration from embryogenic calli the same genotype (BB-6) also performed the best results (19.25%) in MS medium supplemented with 1.5 mg/l BAP + 30 g/l sucrose. Analysis of variance (ANOVA) showed highly significant differences among the media and the genotypes.

Key words: Barley, Callus, Genotypes, Plant growth regulators, Regeneration

Introduction

Somatic embryogenesis is a development process of cells, which resembles morphologically zygotic embryogenesis (De Silva et al. 2009). It is an important pathway for regeneration of plants from cell culture system and a method commonly used in a large scale production of plants (Gniech-Karasawa 2017). Steward et al. (1958) described the asexual embryogenesis (somatic embryos) in carrot, which provided a powerful technique for the mass production of artificial embryos. There are some reports on plant regeneration via somatic embryogenesis in various crop plants (Mamun et al. 2002, Neto et al. 2003, Paul et al. 2013, Rahman et al. 2015, Saha et al. 2017). Many workers have emphasized somatic embryogenesis as a preferred method for genetic improvement and multiplication of valuable germplasm of a number of woody perennials (Gupta and Durzan 1987, Bhansali 1990, Islam and Bhattacharjee 2015). The totipotent character of plant cells that retains its nucleus has the ability to regenerate entire new plant by somatic embryogenesis (SE) or organogenesis (Fortes and Pais 2000). Since somatic embryo cultures often originate from a single cell, it is an ideal system for induction of mutations as it helps in preventing chimeras. The rate of somatic embryo germination is very poor, which has become major hurdle for large-scale plant multiplication of desirable induced mutants (Dahleen and Bregitzer 2002, Lazaridou et al. 2011). Siddique et al. (2014) reported that Bangladeshi indica rice varieties *viz*. BR10, BRRI dhan32 and BRRI dhan47 produced high frequency of callus induction through somatic embryogenesis. The multiplication of true type plants through somatic embryogenesis is very much helpful in propagating elite and new genotypes in shorter periods of time.

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Maximum barley cultivars are often considered as less responsive to tissue culture due to poor callus induction, low frequency of embryogenesis and lesser percentage of plant regeneration (Chauhan and Kothari 2004, Morshed et al. 2014, Haque and Islam 2015). Plant regeneration in barley through *in vitro* culture is highly genotype dependent reported by Castillo et al. (1998) and Han et al. (2011). Therefore, screening for highly responsive *in vitro* genotypes is very important for advance biotechnological work in barley. The process of somatic embryogenesis is not only important for the production of plants and secondary products, but also important for the transgenic plants development with desire characters. Somatic embryogenesis also plays an important role in clonal propagation. When integrated with conventional breeding programs and molecular and cell biological techniques, somatic embryogenesis provides a valuable tool to enhance the pace of genetic improvement of commercial crop species (Stasolla and Yeung 2003). The main objective of this study was to improve somatic embryogenesis as well as screening of suitable barley genotypes in Bangladesh using mature embryos.

Materials and Methods

Mature seeds of six barley genotypes *viz*. BARI barley-1 (BB-1), BARI barley-2 (BB-2), BARI barley-3 (BB-3), BARI barley-4 (BB-4), BARI barley-5 (BB-5) and BARI barley-6 (BB-6) were considered as plant materials for this study.

Sterilization and inoculation

Seeds were collected from Bangladesh Agricultural Research Institute (BARI), Gazipur, Bangladesh. Seeds were sterilized with 70% (v/v) ethanol for 1 minute and washed three times with sterile distilled water then treated with 0.12% NaOCl for 30 minutes followed with three washes with sterile distilled water under aseptic condition. After disinfection, mature seeds were cultured in MS (Murashige and Skoog 1962) medium for callus induction.

Callus induction

For primary callus induction, MS medium supplemented with different concentrations and combination of 2,4-D, BAP, L-proline, casein hydrolysate and 3% sucrose (w/v) are shown in Table 1. Under this study the six barley genotypes and twelve (12) media combination were used as per the PGRs and other components. The cultured petri dishes were incubated in the dark at 25±2°C. After four weeks of culture initiation data were recorded on the basis of number of mature embryos induction.

$$\text{Percentage of callus formation} = \frac{\text{Mature embryos formed callus}}{\text{Total number of embryos cultured}} \times 100$$

Friable and compact calli were assumed as potentially embryogenic (considered as effective callus) and were selected for maintenance and regeneration.

Table 1. MS medium along with various concentrations and combinations for PGRs, L-proline and CA for callus induction of barley genotypes

Medium	2,4-D (mg/l)	BAP (mg/l)	L-Proline (mg/l	Casein hydrolysate (CA) (mg/l)
MS + CIM_1	1.0	-	100	150
MS + CIM_2	1.0	-	200	300
MS + CIM_3	1.0	-	300	450
MS + CIM_4	2.5	0.1	100	150
MS + CIM_5	2.5	0.1	200	300
MS + CIM_6	2.5	0.1	300	450
MS + CIM_7	4.0	-	100	150
MS + CIM_8	4.0	-	200	300
MS + CIM_9	4.0	-	300	450
MS + CIM_{10}	5.5	0.2	100	150
MS + CIM_{11}	5.5	0.2	200	300
MS + CIM_{12}	5.5	0.2	300	450

Embryogenic callus formation

After 4 weeks of culture, primary callus of three barley genotypes (BB-1, BB-3 and BB-6) were separated from explants and transferred them to CIM_4 (MS + 2.5 mg/l 2,4-D + 0.1 mg/l BAP + 100 mg/l L-proline + 150 mg/l casein) for embryogenic callus development. Data were recorded after 3 weeks of incubation and percentages of embryogenic calli (EC) were evaluated.

Plant regeneration

Under this study three barley genotypes (BB-1, BB-3 and BB-6) as well as nine different concentration and combinations of MS media *viz.* RM_1 (MS + 0.5 mg/l BAP + 20 g/l sucrose), RM_2 (MS + 1.0 mg/l BAP + 20 g/l sucrose), RM_3 (MS + 1.5 mg/l BAP + 20 g/l sucrose), RM_4 (MS + 2.0 mg/l BAP + 20 g/l sucrose), RM_5 (MS + 0.5 mg/l BAP + 30 g/l sucrose), RM_6 (MS + 1.0 mg/l BAP + 30 g/l sucrose), RM_7 (MS + 1.5 mg/l BAP + 30 g/l sucrose), RM_8 (MS + 2.0 mg/l BAP + 30 g/l sucrose) and RM_9 (MS + 1.5 mg/l BAP + 40 g/l sucrose) were used and evaluted their regeneration efficiency. In all cases, 2-3% (w/v) sucrose was used as carbon sources. Cultures were maintained at 25°C with 16/8h (light/dark) and regeneration frequency was evaluated after 4 weeks of incubation in regeneration medium. Regenerated shoots were transferred to half-strength of MS medium that supplemented with 20 g/l sucrose and 2.0 mg/l NAA for rooting. The well-rooted plants were transferred into pots.

Data recording and statistical analysis

Each treatment contained three replications and the whole experiment was repeated three times. Statistical analysis was performed using SPSS software (version 16). Data were evaluated on the basis of primary callus induction and regeneration among the studied genotypes and evaluated those parameters by one-way analysis of variance (ANOVA). The significance of differences and comparisons between the mean values were determined by least significant difference (LSD) formulation at 5% level.

Results

For primary callus induction six barley genotypes (BB-1, BB-2, BB-3, BB-4, BB-5 and BB-6) were cultured and evaluated their efficiency using twelve different combinations of PGRs in addition with MS medium. The results indicated that BB-6 showed highest (38.17%) percentage of callus followed by BB-3 (30.11%) and BB-1 (24.04%). Whereas, BB-2 showed very low (7.65%) and BB-4 and BB-5 showed no callus induction (Fig. 1). The compact and friable calli were transferred to regeneration medium and evaluated its regeneration efficiency (Fig. 2). Analysis of variance (ANOVA) showed significantly higher results between the genotypes and culture combinations (Table 3).

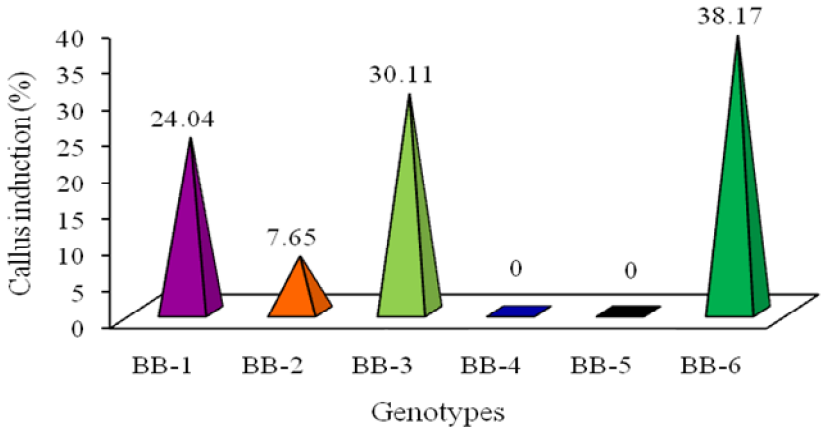

Fig. 1. Primary callus induction responses of mature embryos in six barley genotypes.

MS medium (CIM_4) containing 2.5 mg 2,4-D, 0.1 mg BAP, 100 mg/l L-proline and 150 mg/l CA was found to be very effective for embryogenic callus induction. The percentage of embryogenic callus was 27.49%, 20.66% and 14.89% in BB-6, BB-3 and BB-1, respectively (Fig. 3). The genotype BB-6 showed the highest frequency of embryogenic callus compared with BB-3 and BB-1. In this case nodular, heart and torpedo shaped embryogenic calli gave better green plants than others (Fig. 2).

The media formulation of RM_7 (MS + 1.5 mg/l BAP + 30 g/l sucrose) showed significantly better results for regeneration than others. The highest regeneration percentages were recorded for BB-6 (19.25%), BB-3 (13.33%) and BB-1 (9.72%) in RM_7 mdium (Table 2). On the other hand, RM_1 (MS + 0.5 mg/l BAP + 20 g/l sucrose) showed lowest regeneration (4.48% for BB-6 and 3.80% for BB-3). It was observed that the genotype BB-6 showed good regeneration (9.26%) in terms of average number of plants per somatic embryos. Analysis of variance (ANOVA) showed highly significant differences among the media and the genotypes (Table 3). Regenerated plants were transferred to rooting medium. Then after acclimatization well-rooted plants were transferred to the soil.

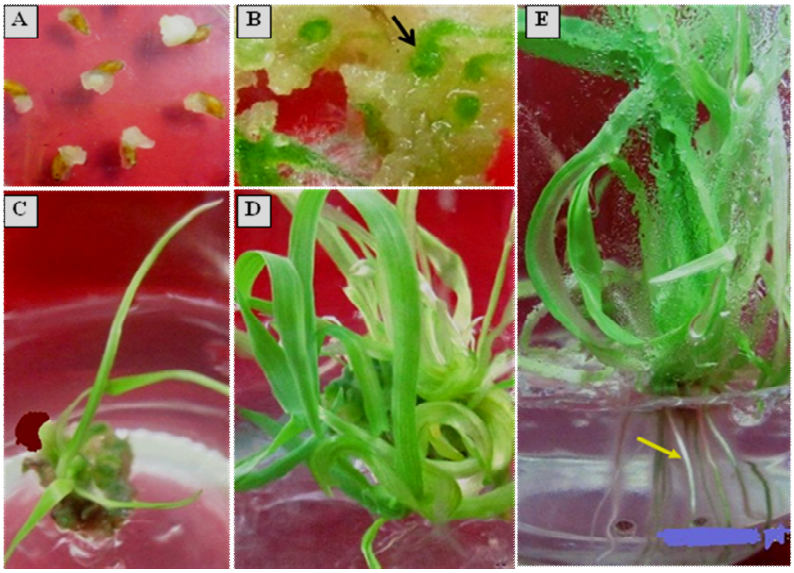

Fig. 2 (A-E). Somatic embryogenesis and plant regeneration in barley. A) Callus derived from seeds after 1 week of culture initiation, B) Embryogenic callus after 5 weeks, C) Initiation of shoots after 7 weeks of culture, D) Shoot developments after 9 weeks of culture, E) Regenerated plants with good roots and shoots after 11 weeks of culture.

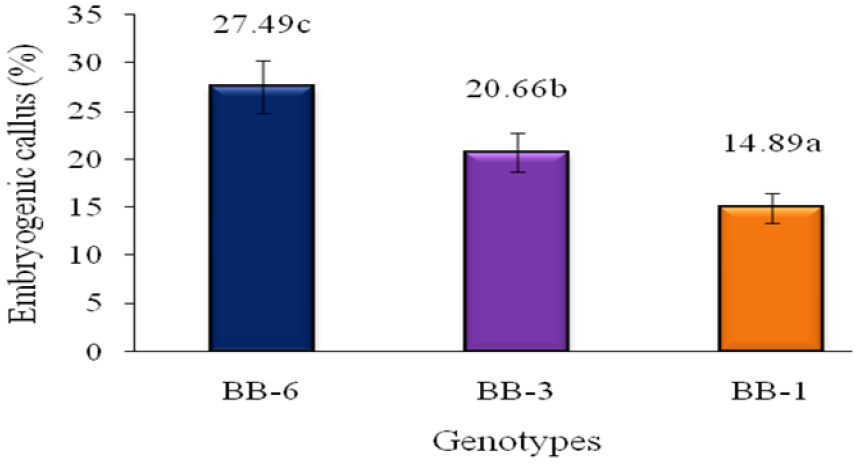

Fig. 3. Frequencies of embryogenic calli derived from mature embryos of three barley genotypes.

Table 2. Effect of nine different RM medium on regeneration efficiency using three barley genotypes

Genotypes	Medium	No. of embryogenic calli (cultured)	No. of calli response to regeneration	% of regeneration (Mean ± SE)
BB-6	RM_1	156	7	4.48 ± 0.64a
	RM_2	135	13	9.62 ± 1.95bc
	RM_3	135	14	10.37 ± 1.48c
	RM_4	135	10	7.40 ± 1.95b
	RM_5	165	10	6.06 ± 1.60b
	RM_6	126	17	13.49 ± 2.09d
	RM_7	135	26	19.25 ± 1.95e
	RM_8	120	9	7.5 ± 1.44b
	RM_9	135	7	5.18 ± 0.74ab
	Mean	**138.00**	**12.56**	**9.26**
BB-3	RM_1	105	4	3.80 ± 0.95a
	RM_2	75	5	6.66 ± 1.33b
	RM_3	84	7	8.33 ± 1.19bc
	RM_4	96	5	5.20 ± 1.04ab
	RM_5	99	4	4.04 ± 1.01a
	RM_6	78	8	10.25 ± 1.28c
	RM_7	60	8	13.33 ± 1.66d
	RM_8	66	4	6.06 ± 1.51ab
	RM_9	102	5	4.90 ± 0.98ab
	Mean	**85.00**	**5.56**	**6.95**
BB-1	RM_1	24	0	0
	RM_2	66	2	3.03 ± 1.51ab
	RM_3	84	4	4.76 ± 1.19b
	RM_4	60	2	3.33 ± 1.66ab
	RM_5	78	2	2.56 ± 1.28ab
	RM_6	61	4	6.34 ± 1.58bc
	RM_7	72	7	9.72 ± 1.38c
	RM_8	102	4	3.92 ± 0.98ab
	RM_9	126	2	1.58 ± 0.79a
	Mean	**74.78**	**3.00**	**3.92**

Different letters of mean values in the same column indicate significant differences within culture combinations (LSD test, $p<0.005$).

Table 3. Analysis of variance (ANOVA) subjected to primary callus induction for two genotypes and plant regeneration for three barley genotypes

	Variable	Source of variation	df	Mean sum of square	F. value
Embryogenesis	Callus induction	Genotype	5	85.26	18.14**
		Culture combination	11	41.08	19.57**
		Genotype × culture combination	11	6.23	-
	Plant regeneration	Genotype	2	64.69	49.02**
		Media formulation	8	37.28	28.26**
		Genotype × media formulation	16	1.31	-

** = significant at 1% level of probability.

Discussion

Plants regeneration is very essential for establishing a successful tissue culture system. In case of tissue culture for all crops do not show equal regeneration ability. Sometimes it is very difficult to culture and regenerate agronomically important crops (Puhan and Siddiq 2013). Somatic embryogenesis is a multi-step regeneration process starting with formation of pro-embryogenic masses, followed by somatic embryo formation, maturation and plant regeneration (Arnold et al. 2002, Sharmin et al. 2014). The difference in the composition of culture medium and the concentrations of hormones affect the callus induction and regeneration ability of barley and other plant genotypes (Tariq et al. 2008, Haque and Islam 2014). As auxin 2,4-D is very important and required for the production of somatic embryogenesis in cereal crops (Armstrong et al. 1987, Nasircilar et al. 2006). Somatic embryos are formed on nutrient medium with a reduced 2,4-D concentration is reported by Delporte et al. (2001). Furthermore, genotype variation also plays a vital role in callus initiation, proliferation and even regeneration in barley (Gubišová et al. 2012) and rice (Khanna and Raina 1998).

In this study, twelve different concentration and combinations of PGRs were used with MS medium and found that significant differences between media components. It was observed that 4.0 mg/l 2,4-D + 200 mg/l L-proline and 300 mg/l casein hydrolysate promoted callus induction in barley. However, increasing amount of 2,4-D from 1 mg/l to 4.0 mg/l showed significantly highest percentages of callus induction. The culture combination of CIM_8 contained MS + 4.0 mg/l 2,4-D +200 mg/l L-proline + 300 mg/l casein hydrolysate gave best primary callus induction when used mature embryos as explants. Chernobrovkina et al. (2004) studied

with addition of L-proline (160 mg/l) showed negative impact on the *in vitro* embryo culture in barley. Aguado-Santacruz et al. (2011) demonstrated that 2 mg/l of 2,4-D showed better callus induction. Whereas, in addition of amino acid with higher concentrations (230 mg/l) did not show any callus induction. Till several protocols for *in vitro* callus culture of barley and other crops have been successfully developed by Lupotto (1984), Bregitzer (1992), Castillo et al. (2000), Chauhan and Kothari (2004), Asakavičiūtė and Pašakinskienė (2006), Gubišová et al. (2012), Haque and Islam (2014), Mrízová et al. (2014), Haque and Islam (2015). However, only a few barley genotypes have been identified that possesses good regeneration capacity (Lemaux et al. 1999, Aguado-Santacruz et al. 2011). Suitable barley response to *in vitro* culture with highest regeneration potential and correct explants as well as their proper developmental stage to be used as genetic transformation (Chang et al. 2003, Kasha 2007). It was reported that mature (Sharma et al. 2005, Yadav et al. 2011) and immature embryos (Walmsley et al. 1995, Haque and Islam 2015) were suitable explants for somatic embryogenesis in barley and other cereal crops. Under this study, successfully induced callus from mature embryos of some barley genotypes using MS medium. The medium was supplemented with different concentrations of 2,4-D, L-proline and casein hydrolysate. The results obtained in this study are quite similar with the report of Ganeshan et al. (2003), who successfully induced callus from mature embryo of barley. They used similar components in the medium (2,4-D, L-proline and casein hydrolysate) but the concentration was different with the present findings.

Various concentration and combination of auxins, cytokinins play an important role for embryogenic callus induction in barley reported by Serhantova et al. (2004). The present results showed that MS + 2.5 mg/l 2,4-D + 0.1 mg/l BAP + 100 mg/l L-proline + 150 mg/l casein hydrolysate was adequate for embryogenic callus formation. Walmsley et al. (1995) stated that 2.0 mg/l of 2,4-D was suitable for initiation of embryogenic callus in barley. Bregitzer et al. (1998) reported that the formation of embryogenic callus in various barley genotypes was depended on 2,4-D concentration, and 2-3 mg/l was adequate in most of the cases. Amali et al. (2014) demonstrated that the addition of L-proline considerably improved the somatic embryo formation in MS medium containing 2.5 mg/l 2,4-D and 500 mg/l casein hydrolysate in sorghum. Casein hydrolysate can be used as a relatively cheap source of a mixture of amino acids (Slater et al. 2003). In addition of amino acids in the medium serve as a source of reduced nitrogen required for plant metabolism and growth. The present study revealed that the addition of casein hydrolysate to the medium enhanced embryogenic callus formation in barley.

In this study plant regeneration efficiency were tested and for that nine regeneration media were used that contained MS basal medium with different concentrations of BAP and sucrose. The present results demonstrated that regeneration formulation of RM_7 (MS + 1.5 mg/l BAP + 30 g/l sucrose) functioned better than others. This result agreed well with previous works where the MS medium and BAP were successfully used in barley (Aguado-Santacruz et al. 2011). There are some reports on the effect of sucrose was scrutinized on induction of plant (Shah et al. 2014). A similar type of result was found by Lee et al. (2012). They reported that sucrose has been commonly used at the concentration of 20 and 30 g/l as a carbon source in tissue culture medium. But they have not found any combined effect of different concentrations of BAP and sucrose for regeneration purpose. In this case BB-6 showed particularly high stability in callus induction over the different culture combination and higher regeneration compared to BB-3 and BB-1. However, this results proven that embryogenic callus formation and plant regeneration ability are depends using a suitable genotype.

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