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RESEARCH ARTICLE

High frequency plant regeneration from immature embryos of an elite barley cultivar (*Hordeum vulgare* L. cv Baegdong)

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Abstract: An efficient plant regeneration system was developed for *Hordeum vulgare* L. cv Baegdong - an important high yielding Korean cultivar. This cultivar is resistant to *Fusarium* and a few other fungi but susceptible to Barley Yellow Mosaic Virus (BaYMV) and Barley Mild Mosaic Virus (BaMMV). The protocol was based on a series of experiments involving the sizes of immature embryos and the culture media. The embryo size is found to be critical for the establishment of callus. Embryos of 1.6-2.0 mm size showed the highest ability to produce callus capable of regenerating green plants. The auxins picloram and dicamba proved effective in inducing callus from immature embryos. 2.5 mg L^{-1} dicamba and 4.0 mg L^{-1} picloram in Murashige and Skoog (MS) medium was optimum for the induction of primary callus. The induced primary callus was loose and friable which ultimately developed into creamy white and compact callus after transferring into the fresh medium. Multiple shoots were induced in the ½MS medium supplemented with 6.0 g L^{-1} maltose, 20.0 g L^{-1} sorbitol, 0.5 mg L^{-1} 2, 4-D and 1.0 mg L^{-1} kinetin and the rate was 6.5 shoots per embryo. Regenerated plants were hardy and developed roots rapidly in the medium containing 0.2 mg L^{-1} Indolebutyric acid (IBA). This efficient plant regeneration system provides a foundation for generating transgenic plants of this important barley cultivar.

Keywords: Barley embryo, embryo size, *Hordeum vulgare*, plant regeneration, primary callus.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is an important cereal crop used for malting, brewing, distilling and as an animal as well as a human food. Barley has been the subject of extensive genomics research and is considered a model for the Family Triticeae. Genetic transformation is a key tool for genetics research which can be of agronomic importance. Low recovery of green plants from barley callus cultures is a major problem limiting the efficiency of generating transgenic barley. Although both biolistics and *Agrobacterium*-mediated methods have been successfully applied for genetic transformation of barley, most work has been performed on a single model barley cultivar, 'Golden Promise'[1-3]. Immature embryos are presently being used as the explant[1,4] and many related factors such as the size of the embryo, and growth regulator combinations involved in successful regeneration have been studied in detail. Immature embryos are suitable explants for genetic transformation but this requires a consistent supply of stock barley plants in growth chambers or greenhouses. The above protocol has a poor success rate when applied to other barley cultivars. Selecting the proper stage of maturity may be one of the limiting factors when using immature embryos. There are records of successful shoot and bud induction from calli derived from mature embryos in other cereals such as oat[5] and rice[6]. Although there are reports on the use of mature embryos for regeneration of barley[7-9], the success rate has been very low. Barley regeneration through mature embryo derived callus is also cultivar specific[9]. The purpose of this study was to determine optimum conditions for regenerating plants from immature embryos of 'Baegdong' barley, and to develop an efficient regeneration protocol that can be used for genetic transformation and other fundamental studies. The effect of the size of the embryos, basal medium and plant growth regulators on callus induction, maintenance and regeneration of green plants were also studied.

METHODS AND MATERIALS

Seeds were soaked in water for 1h and then surface sterilized with 10% Clorox (5.25% v/v sodium hypochlorite) for 20 min with gentle shaking followed by 70% v/v alcohol for 1 min each and three successive washings in sterile

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distilled water. Embryos were dissected and cut along the longitudinal axis before being transferred onto callus initiation medium (CIM) and maintained for 2–4 weeks at 25°C and a 16-h day regime. Unless otherwise stated, all the culture media were prepared with 30.0 g L^{-1} sucrose and 8.0 g L^{-1} agar and the pH was adjusted to 5.75 before autoclaving. Dicamba and picloram were filter sterilized before adding to the autoclaved medium.

Table 1: Different growth regulator combinations used with three basal media used

Treatment code	2.4 D (mg L^{-1})	Dicamba (mg L^{-1})	Picloram (mg L^{-1})
T 1	2	0	0
T 2	2	0	2
T 3	2	0	4
T 4	0	2.5	0
T 5	0	2.5	2
T 6	0	2.5	4
T 7	0	5.0	0
T 8	0	5.0	2
T 9	0	5.0	4
T 10	0	0	2

Effects of basal media and plant growth regulators on callus induction: Three types of basal media, Murashige and Skoog medium (MS), Gamborg medium (B5) and Nitsch medium (N6) were used with different concentrations and types of plant growth regulators to examine callus induction from embryos. Embryos were cultured on either MS, B5 or N6 medium without or supplemented with 2.0 mg L^{-1} 2,4-D and picloram (0, 2.5 or 5.0 mg L^{-1}) and dicamba (0, 2.0 or 4.0 mg L^{-1}) in different combinations (Table 1) for 4 wks. Each produced callus was divided into 2–3 mm diameter pieces and transferred to containers with identical fresh medium. There were 30 replicates in each treatment.

Plant regeneration: After 8 wks of subculture in the identical fresh medium (MS + 2.5 mg L^{-1} picloram + 4.0 mg L^{-1} dicamba) for secondary callus production, they were transferred into three regeneration media R1, R2 and R3 where the basal media respectively were N6, MS and ½MS (both macro and micro elements at half strength) supplemented with 2,4 D (0.5 mg L^{-1}) and kinetin (1.0 mg L^{-1}). 6.0 g L^{-1} maltose and 20.0 g L^{-1} sorbitol were also added to the media. There were 30 replicates in each treatment. After 6 wks of incubation, morphogenic changes in each callus was observed using light and scanning electron microscope (SEM).

Effects of embryo size on callus induction and regeneration: The effect of the size of the embryo (1.0–1.5, 1.6–2.0, 2.1-2.5 and 2.6-3.0 mm) on callus induction and plant regeneration were studied. Seeds were collected after 18 to 22 d of pollination. Embryos were sorted randomly and there were 30 replicates for each size class. The embryos of each size class were cultured on callus induction medium (CIM) - (MS + 2.5 mg L^{-1} dicamba + 4.0 mg L^{-1} picloram)-for 6 wks, and then transferred to

Table 2: Morphogenic changes in the secondary calli on culture media R1-R3. Observations after 6 weeks of incubation

Medium	Mean callus degeneration	Percentage shoot initiation	Percentage root initiation	Mean no. of shoots/callus
R1	9.0 ± 1.1	2	12	11.0 ± 0.5[a]
R2	-	14	8	11.5 ± 1.6[a]
R3	-	75	8	26.5 ± 1.4[b]

Means within columns with the same letter are not significantly different ($p<0.05$).

Table 3: Effects of the size of embryos on regeneration frequency of cv Beagdong barley (data obtained after 4 weeks in regeneration medium)

Size of embryos (mm)	No. of embryos producing primary callus	No. of embryos producing secondary callus	Mean no. of shoots/ embryo
1.1–1.5	30.0	25.0	4.8±0.8[b]
1.6–2.0	30.0	28.0	6.4±1.4[a]
2.1–2.5	30.0	26.0	2.6±1.1[c]
2.6-3.0	26.0	2.0	0.8±1.5[d]

Means within columns with different letters are significantly different ($p<0.05$).

regeneration medium (R3) incubated at 25°C under white fluorescent light at approximately 40 μE m^{-2} s^{-1} in a 16-h day. Number of embryos producing primary and secondary calli and also the mean number of shoots per embryo were recorded after 4 wks.

Rooting of regenerated plants: Regenerated shoots were transferred to rooting medium when the leaves were about 1 cm in length. Rooting medium consisted of half-strength MS medium supplemented with 0.2 mg L^{-1} IBA, 20.0 g L^{-1} sucrose, and 6.0 g L^{-1} agar. After 3–4 wks, plantlets with well-developed roots were transferred to soil.

Statistical analysis: Completely Randomised Design was used in all experiments. Multifactor ANOVA was performed. To compare the treatment means, restricted LSD (Least Significant Diffrerence) was performed. Tukey test was not applied when individual error rate was very low.

RESULTS AND DISCUSSION

Effects of basal media and plant growth regulators on callus induction

Both 2, 4-D, dicamba and picloram induced, vigorously growing, friable calli (primary calli). Subsequently, milky white, compact and nodular shaped calli (secondary calli) were formed on the surface of the primary calli. The same type of primary and secondary calli have been observed in some of the barley cultivars tested[9]. SEM indicated that, cells in the primary calli were elongated while the cells from secondary calli were globular shaped and compact. Tips of the elongated cells form a globular shaped sack at the stage of shoot initiation. All treatments in MS basal medium showed high degree of callus formation (0.25 – 0.42 g). B5 basal salts showed low response in callus production. No callus induction was observed in most of the treatments where basal medium was B5. However, in T3, T4 and T5 a very low level (0.18 – 0.21 g) of callus induction was observed. Low concentration of dicamba (2.5 mg L^{-1}) and high concentration of picloram (4.0 mg L^{-1}) were found to be the best combination for callus induction in both MS and N6 media (0.42 g – 0.35 g respectively) (Figure 1). Higher concentrations of growth regulators were known to result in a greater possibility of somatic mutations[10]. Plant regeneration from transgenic barley calli was difficult when 2, 4-D was used[3]. Therefore, use of dicamba and picloram may be a better choice for transgenic barley plant regeneration and the optimal concentrations would be 2.5 mg L^{-1} dicamba and 4.0 mg L^{-1} picloram for the Baegdong variety.

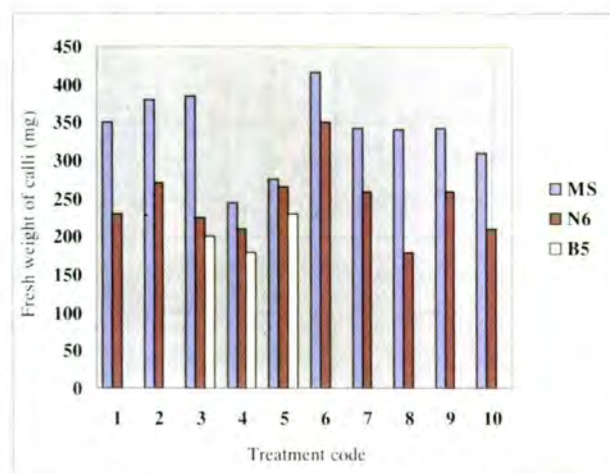

Figure 1: Fresh weight of calli in the presence of different growth regulators after 5 weeks

Plant regeneration

Some of the calli transferred into R1 where the basal medium was N6 degenerated and turned yellowish brown after 2 weeks and the electron micrographs indicated that the cells were deformed. Composition of the basal medium has a significant effect over the percentage shoot initiation from calli (2% in N6, 14% in MS and 75% in ½MS which represents R1, R2 and R3 respectively). Mean number of shoots per callus was highest (26.5±1.4) in ½MS supplemented with 0.5 mg L^{-1} 2, 4-D and 1.0 mg L^{-1} kinetin (R3). Although the basal medium constituents are different in R1 (N6) and R2 (MS), the mean number of shoots per callus was almost the same (11.0±0.5 and 11.5±1.6 respectively) (Table 2). Some of the researchers have observed albino plants[4,7] in tissue cultured barley but no albino plants were observed in the present study. Kinetin and 2,4-D in ½MS medium significantly improved the regeneration efficiency from mature embryos of barley but the concentrations required were cultivar specific[9].

Effects of embryo size on callus induction and regeneration

When embryos were cultured on CIM, callus growth was initiated in about 5 d. Smaller embryos (less than 2.5 mm) produced significantly high percentage of compact calli than larger embryos (larger than 2.6 mm). Embryos of 1.6-2.0 mm size resulted in the highest formation of secondary calli (28) and also generated the highest number of green plants per embryo (6.4±1.4). Although most of the larger embryos (2.6 - 3.0 mm) produced calli (26), only few of them formed compact secondary calli. These were very hard and regeneration rate was very low (0.8±1.5 plants per embryo) (Table 3). The calli derived from the 1.6–2.0 mm embryos were very soft and white or light coloured during the first week. They grew quickly and gradually became more compact and yellowish. More than 80%

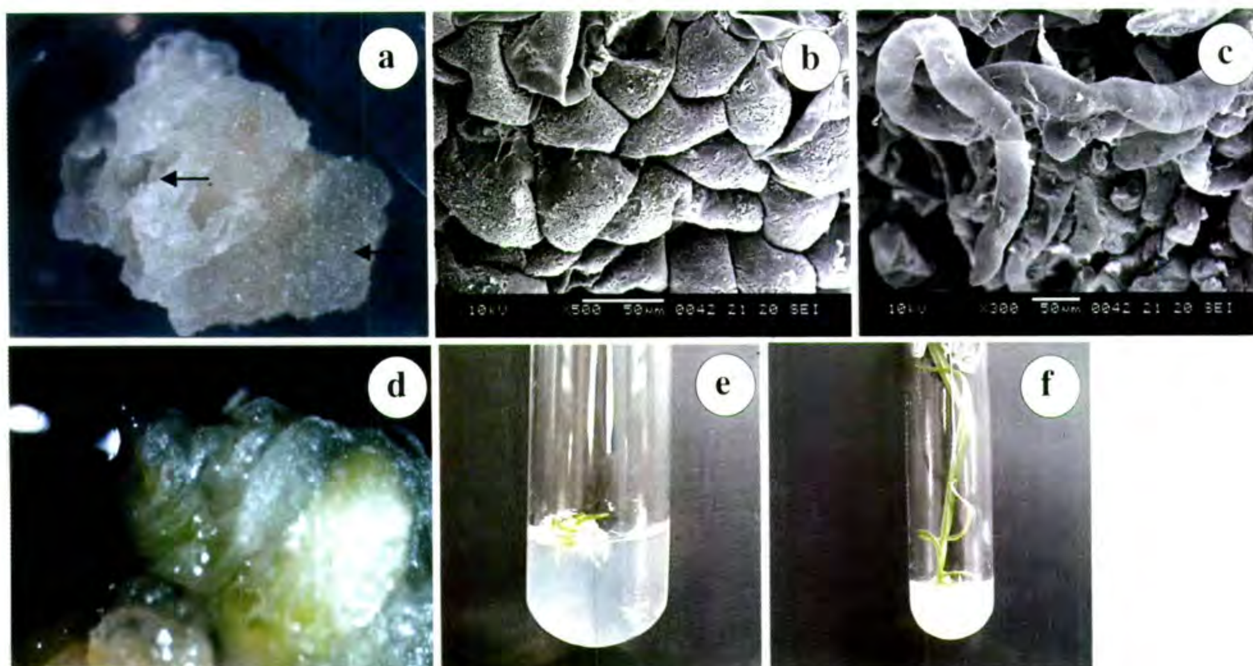

Figure 2: (a) Primary (left) and secondary (right) calli, (b) Elongated cells of primary callus (SEM), (c) Globular cells in secondary callus (SEM), (d) Shoot initials in secondary callus (e) Rooting and (f) Fully grown plantlet

became compact within 3 weeks and these calli initiated shoots in 2–3 weeks. In this experiment, it was observed that the embryos of 1.6–2.0 mm had the highest regeneration potential. Some cereal crops regenerate from calli derived from mature embryos, and the optimal size of embryos varies with plant genotype or species[11]. Developmental stage of the immature embryos was reported to be a crucial factor for *in vitro* regeneration in barley[12] and other cereal crops[13-15].

Rooting of regenerated plants

When multiple shoots grown on regeneration medium were divided and transferred to rooting medium (half-strength MS basal medium with 0.3 mg L^{-1} IBA), thick white roots developed in about 1 week. Plantlets with well-developed roots were successfully transferred to soil.

This study reveals that there is an effect of the size of the immature embryo on *in vitro* regeneration of Baegdong barley and that picloram and dicamba play an important role in callus induction. Changing the carbohydrate source to a mixture of maltose and sorbitol and also reducing the concentration of basal salt mixture favoured shoot induction from calli.

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