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COMPARING THE EFFECT OF EMBRYONIC AXIS REMOVAL ON CALLUS INDUCTION EFFICIENCY FROM TWO BARLEY VARIETIES CULTURED WITH EXTRA COPPER

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ABSTRACT

Embryonic axis-based callus induction and plant regeneration performance of two spring barley (*Hordeum vulgare* L.) varieties *viz* 'Golden promise' and 'Clansman' were compared *in vitro*. We have assayed different combinations of nutrient media and growth regulators to induce callus and plant regeneration from explants of isolated immature embryo of barley. The best results for callus induction were obtained with immature embryo in MS medium supplemented with 2.0 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) in addition to 16.25 mg/l $CuSO_4.5H_2O$. Shoot regeneration from barley calluses was the highest (61.0% for cv. Golden Promise) on callus induction media with 0.5 mg/l BAP (6-Benzylaminopurine). For further development of these regenerated plants hormone free MS medium was used. The *in vitro* regenerated plantlets were transplanted into soil and grew up to maturity like the control plants. The callus induction frequencies for both varieties were almost the same; however, germination ability was slightly higher for immature embryos of Golden promise than Clansman.

Keywords: *MS medium, Golden promise, Clansman, Barley, 2,4-D, BAP*

Introduction

Barley (*Hordeum vulgare* L.) is a grass species under tribe Triticeae which has been classified as one of the oldest domesticated crops. It has been widely grown in marginally productive soils across the world. Currently, as the fourth most significant cereal, about 147 million tons of barley is produced from 47 million hectares of land extending from 106 countries with average productivity of about 3.0 ton/ha (FAO 2017). In general, it has been utilised globally for fodder, malt, and other cultural centric purposes. Due to its versatile growing traits, barley productivity and yield are more consistent than any other economically important cereal (Newton *et al.* 2011). With 142 million tonnes of barley being utilised last year, its contribution in crop rotation systems

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and as a feed crop within meat industries is highly associated with food security (Mascher *et al.* 2016). The *in vitro* culture of cereal crops has received much attention as a crop enhancement method and an essential aspect of developing functional genomics resources. A highly efficient barley micropropagation and transformation mechanism are required to meet the ever-increasing demand for barley in the food and beverage industries. Unfortunately, barley is one of the recalcitrant cereal crops, with only a limited number of explants available for *in vitro* regeneration and genetic transformation (Suo *et al.* 2021). The donor plant, various explants, different media compositions, genotype and environmental factors play a significant role in the regeneration of barley explants (Rashad *et al.* 2020).

The discovery of particular genotypes capable of rapid callus development and high rates of plantlet regeneration is a critical step in applying tissue culture techniques to any crop plants. Golden Promise is a traditional spring barley cultivar from the United Kingdom, which has become the primary source of academic interest in recent years due to its great genetic transformability. The best shoot recovery ability from callus transformed with Golden Promise most successful (Hensel *et al.* 2008). Although some other cultivars have been tested and utilised successfully, the transformation efficiency of Golden Promise is always greater (Lim *et al.* 2018). In most cases, young embryos are chosen as the preferred target explant in barley. The *in vitro* responses of embryos with and without embryonic axes have distinct variances (Han *et al.* 2011). They can be distinguished by their callus growth response, ability to generate roots and shoots and potentiality to regenerate. However, Copper levels above a certain threshold have also been shown to benefit the plant regeneration potentiality in other species (Joshi and Kothari, 2007). This study compared embryonic axis-based callus induction and regeneration potentiality of immature embryos from two barley varieties when supplemented with extra copper.

Materials and Methods

Two barley cultivars: 'Golden promise' and 'Clansman', were sown in the soil in a 25cm^2 pot for eight days and later transferred into a 13cm diameter pot under a 16-h light/8-h dark cycle at 15°C in a growth room with 80% relative humidity and 500 µmol/m2/s' light. Plants were grown up to the seedling stage. The immature seeds were taken as transfer material. The washing steps of seeds were done in three steps; at first, the seeds were kept in water with a few drops of Triton X-100, then washed thoroughly with water and then surfaced sterilized was done in 70% (v/v) ethyl alcohol for 30 seconds, next in 50% sodium hypochlorite for 4 min and rinsed multiple times with sterile distilled water. The immature embryos sized 1.0-2.0 mm was used for regeneration (Fig. 1). First, the embryonic axis was aseptically excised from the immature embryos with fine forceps, then the embryos were placed with the scutellum up down on callus induction (CI) solid medium. After ~3 weeks, the calluses were initiated in CI media and transferred to transition media. Then calluses were transferred to the regeneration medium for shoot and root initiation and maintained for three weeks at 26±1 ºC in a 16 h light (2000 lux), 8 h dark photoperiod. The components of different types of media used for callus induction, transition and regeneration are listed in Table 1. Throughout the tissue culture procedure, additional $CuSO_4.5H_2O$ was added to each culture plates.

Table 1. Media components for callus induction and regeneration of barley.

Callus induction media (CI)	Transitional Media	Regeneration media
4.3gm/L Murashige and Skoog (MS)[a]	2.7 gm/L MS [a]	2.7 gm/L MS [a]
30 gm/L Maltose [a]	20 gm/L Maltose [a]	750 mg/L Glutamine[b]
1.0gm/L Casein hydrosylate [a]	750 mg/L glutathione[b]	100 mg/L Myo-inositol[b]
350mg/L Myo-inositol[b]	100 mg/L Myo-inositol[b]	0.4 mg/L Thiamine-HCl[b]
690 mg/L Proline[b]	0.4 mg/L Thiamine- HCl[b]	3.5 gm/L phytagel [a]
1.0 mg/L Thiamine-HCl[b]	2.5mg/L 2,4-D dichlorophenoxyacetic acid (2,4-D) [b]	-
2.5 mg/L Dicamba[b]	0.1 mg/L 6-BAP[b]	-
3.5 g/L phytagel [a]	3.5 gm/L Phytagel [a]	-
1.25 mg/l $CuSO_4.5H_2O$	165 mg/L NH_4NO_3 [b]	-
'[a]' = Autoclaved at 121°C for 15 minutes '[b]' = Filter sterilized		

Data were collected based on callus induction and plant regeneration. The mean value was calculated using three replications of embryos with or without embryonic axis with standard error (SE). The callus induction rate with extra copper was calculated using this formula: callus induction rate (%) = (number of explants produced callus/total number of explants cultured) × 100%. The regeneration frequency of the explants was calculated as regeneration frequency (%) = (number of regenerated green plants or albinos/total number of calli isolated) ×100% in the regeneration media of both varieties.

Results

A total of 1741 cases of the different sized embryos were isolated in this study. There were no differences in callus growth when the scutellum was placed in contact with the media from the bottom or from the top. There were, however, noticeable disparities in the reactions of embryos at various stages of development. Embryos collected 7–8 days after anthesis developed rapidly into calli. The number of days after anthesis was significant, but the size of the explant had a substantial impact on culture establishment success. Embryo lengths ranging from 1.0 to 1.5 mm were most efficient in inducing rapid callus development while inhibiting premature embryo germination. The size and number of isolated embryos are shown in Table 2. The immature embryos were inoculated in CI medium supplemented with different concentrations of 2,4–D (0.5-2.5 mg/l) for callus induction (Fig.1). Callus development began after 4-6 days of immature embryo culture. The embryos were cultured on the callus induction medium (CI) in two different ways; (1) The embryonic axis was removed, and the embryos were placed on the medium and (2) The embryos were removed from the seeds without removing the embryonic axis and after a few days when the leaves started to grow from the embryos, the leaves were cut to induce callus

growth. The embryos without embryonic axis are denoted by 'AE' and the embryos that were allowed to form leaves are denoted by 'IE' throughout the study. The difference in embryonic axis excision timing had a significant influence on the frequency of callus induction in each embryo culture method. For both cultivars, 'AE' callus showed better growth and formed yellowish, more compact callus, whereas 'IE' embryos formed a less number of whitish and friable callus (Fig. 1 and 2).

Table 2. Size wise distribution of isolated embryos.

Barley variety	No of isolated embryos		
	1.5mm	1.0mm	0.5mm
Golden promise	461	350	242
Clansman	370	320	218
Total	=831	=670	=460
Percentage (%)	47.73%	38.48%	26.42%

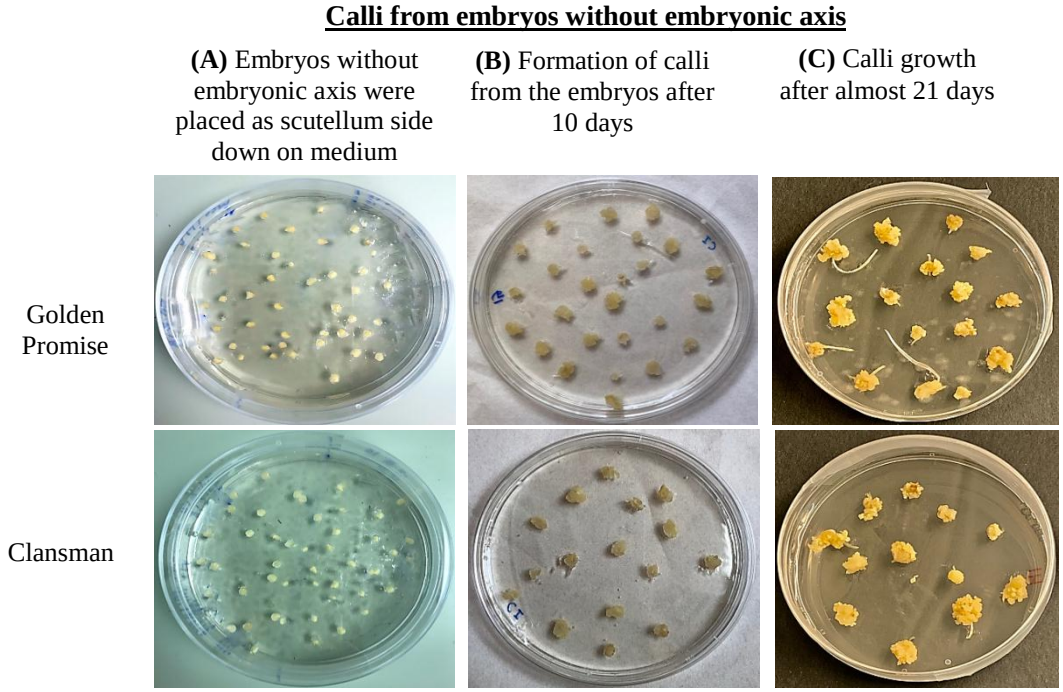

Fig. 1. Callus formation from embryos of Golden promise and Clansman without embryonic axis (AE) were placed as scutellum side down on CI medium. (A)-(C) showing 4 days, 10 days and 21 days old callus formed from 'AE' embryos of Golden promise and Clansman on CI medium.

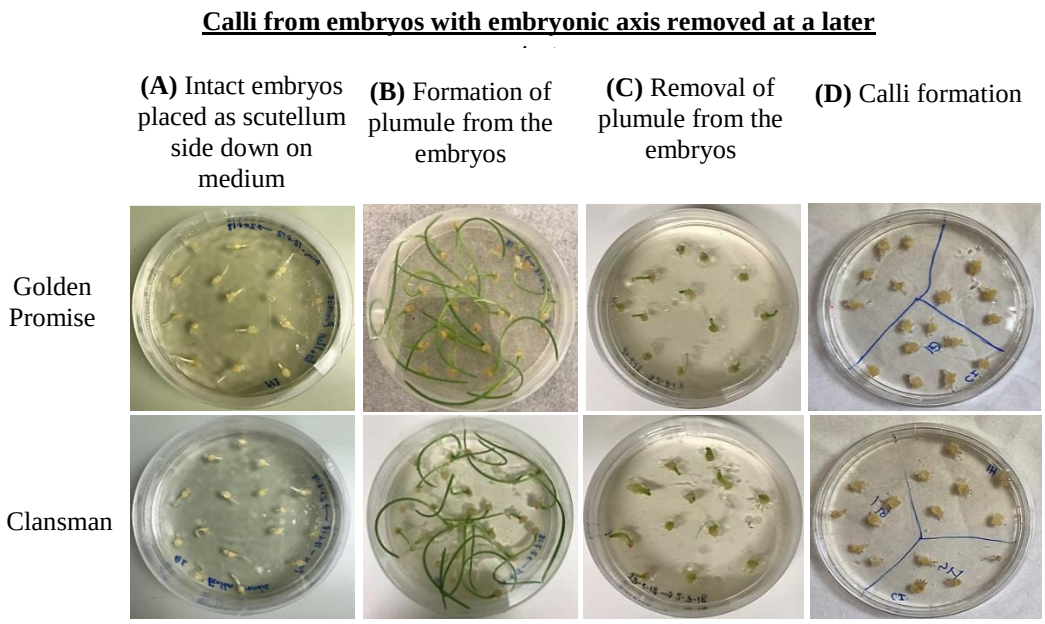

Fig. 2. Callus formation from embryos of Golden promise and Clansman with embryonic axis (IE) were placed as scutellum side down on CI medium. (A)-(D) showing 4 days,1 week, 2 weeks and 4 weeks old callus formed from 'IE' embryos of Golden promise and Clansman on CI medium.

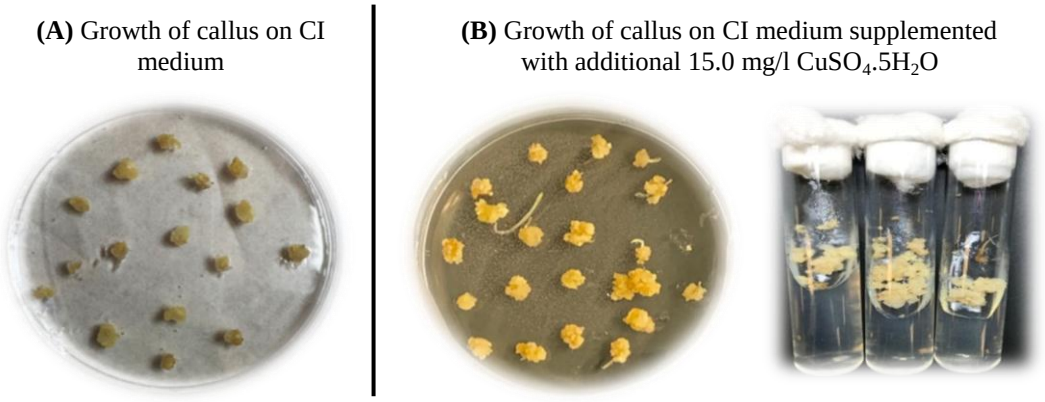

Fig. 3. The effect of extra copper on callus growth. (A) and (B) showing improvement of callus growth derived from immature embryos after 25 days culture on (A) standard CI medium (B) CI medium with additional 15.0mg/l $CuSO_4.5H_2O$

Callus production in both types of embryo cultures reached its peak after 15-21 days of culture on callus induction media. The callus sizes ranged from 4 mm to more than 10 mm, depending on the

concentration of copper. Both 'AE' and 'IE' embryos from two cultivars showed relatively more significant callus production ability in the presence of additional copper (Fig. 3).

Fig. 4 showed the percentage of callus formation by 'AE', and 'IE' embryos of the two varieties supplemented with additional copper. Both varieties generated the highest percentages (75% and 60% of Golden promise and Clansman, respectively) of calli when cultured with an additional $CuSO_4.5H_2O$ while the lowest percentages (58% and 52% of Golden promise and Clansman, respectively) of calli were observed when standard CI medium was used for callus induction (Fig. 4).

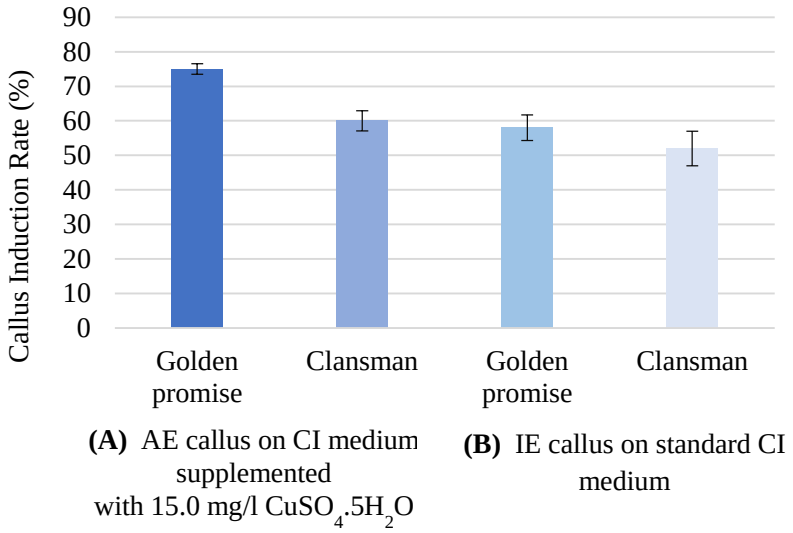

Fig. 4. Callus induction frequency. The left panel shows the increased callus induction frequency of Golden promise and Clansman while the right panel shows the callus induction rate of the two varieties with standard medium. Here, results are represented as mean ± SE, n=3

From the callus induction media, the calli were transferred to the regeneration media for almost 1 month for root and shoot regeneration. The result of Fig. 5 showed the high mean percentage of shoot formation formed by 'AE' embryos of Golden promise and Clansman (61% and 50% respectively) in CI+15.0 mg/l $CuSO_4.5H_2O$. This was followed by the mean percentage of shoots of the 'IE' embryos of the two varieties (58% and 48%) in the standard CI medium.

Initiation of shoot from callus of AE in Golden promise was observed within one week of inoculation (Fig. 6 A & B). Shoots regenerated in this medium (CI with 0.5 ml/l BAP and 15 mg/l $CuSO_4.5H_2O$) from AE were healthy, green and produced a large number of adventitious roots. After development of roots, plantlets of both Golden promise and Clansman were successfully transplanted into small plastic pots containing soil (Fig. 6 C). Following proper acclimation the plantlets were transferred to the field.

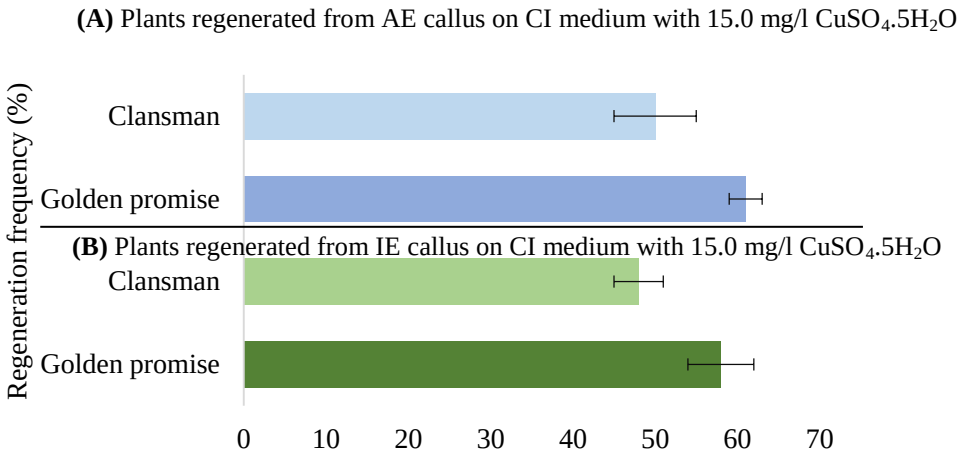

Fig. 5. Regeneration efficiency of AE and IE embryos. (A) and (B) showing regeneration effeciency of AE and IE embryos of Golden promise and Clansman when were tranferred from callus induction medium to regeneration media for root and shoot formation. Here, results are represented as mean ± SE, n=3

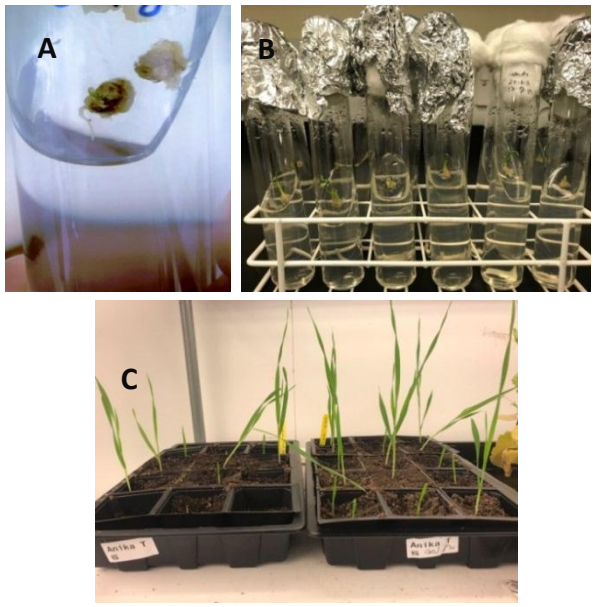

Fig. 6. Regeneration in two varieties of barely namely, Golden promise and Clansman. AE explant from Golden promise showing the initiation of shoots (A and B). Plantlets of Golden promise in soil in green house (C).

The regeneration protocol developed here has been proved reproducible and more or less genotype independent. This protocol may be effectively used for the improvement of different barley cultivars through suitable methods of genetic transformation.

Discussion

The *in vitro* regeneration technique is a potent tool in developing disease-free plants, rapid replication of unique plant genotypes, plant genome transformation, and creation of commercially valuable plant-derived compounds **(**Fehér 2019, Bidabadi and Jain 2020). Different explants and their size from different cultivars of barley have been assessed in the past for their higher callus producing capabilities and quick callus initiation, though most of the studies were confined to the immature embryos from the variety 'Golden promise' (Hensel *et al.* 2008). Previously Chang *et al.* (2003) found that 0.5-1.5 mm sized immature embryos have 100% callus development and better efficiency of plant regeneration than larger embryos which is consistent with our findings. The genotype and explant dependency of tissue culture methods in barley is universal and unavoidable due to the recalcitrant nature of this crop plant (Han *et al.* 2011). The most popular and frequently used barley *in vitro* culture and transformation process was optimized for the explants of the model cultivar Golden Promise (Tingay *et al.* 1997, Harwood 2014). Alternative protocols for several additional barley genotypes, such as the advanced Australian spring barley breeding line WI4330 (Ismagul *et al.* 2014) and hull-less barley, have been refined (Lim *et al.* 2018). However, attempts to alter additional barley varieties using this approach have failed or resulted in low transformation frequencies (Hensel *et al.* 2008).

The effect of genotype on callus induction ability from mature embryo cultures of barley has previously been documented (Akula *et al.* 1999; Bregitzer 1992). The current findings add insight to the existing knowledge about the responses of another barley variety, 'Clansman,' to various *in vitro* cultural growth environments. 'Clansman' variety was almost as efficient as 'Golden promise' in callus induction, and shoot & root proliferation. This variety with potential shoot proliferation and regeneration can be exploited in future tissue culture based genetic improvement study. We assume that it will be valuable to the entire barley research community, but especially to groups working on different available commercial types.

Several authors observed that sometimes immature embryos are also tricky to transform due to a lack of an efficient approach (Dahleen 1999 and Sharma 2005). Many studies were conducted to overcome limits in transformation frequency by enhancing tissue culture procedures to boost regeneration rates (Temel *et al.* 2008, Ikeuchi *et al*. 2016). Other research has concentrated on the modification of explant tissue, as well as the use of a variety of concentrations of growth hormones and culture conditions (Šerhantová *et al*. 2011). We demonstrated an inexpensive approach to study callus induction frequency. The timing of embryonic axis removal and copper supplementation was investigated in this study to boost regeneration rates significantly. Here we used embryos as a potential source of explants, and they were put on the callus induction media in two different ways. The embryonic axis was removed from the embryo and cultured on the callus induction media in the first process.

On the other hand, the second procedure involved the intact embryo cultured on the callus induction media. This study's standout finding is the successful utilization of both ways of culturing embryos as a source for the formation of calli and capable of shoot growth in combination with excessive copper. Dahleen (1995) and Bartlett *et al.* (2008) described improved

callus formation and plant regeneration in barley by employing higher quantities of copper. An experiment like this one, which selects for regulators that enhances the regeneration capacity of the explants, will aid in identifying the variables that influence *in vitro* response of different tissues.

Conclusion

Plant tissue culture from mature and immature embryos of Barley is a critical tool for a variety of functional genomics investigations as well as crop enhancement in the future. The demand for effective crop transformation systems increases and new genome editing technologies contribute to that demand. Based on *Agrobacterium*-mediated inoculation of immature embryos, we compared the callus induction and regeneration frequency for the two barley varieties viz. Golden Promise and Clansman. Embryo culture from these two varieties can be used in the future for crop improvement, secondary metabolite production, and various strategies for inducing genetic interference.

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