Turk J Biol
26 (2002) 175-180

Embryo Culture in Barley (*Hordeum vulgare* L.)*

Betül BÜRÜN, Esin ÇOBAN POYRAZOĞLU
Muğla University, Faculty of Science and Arts, Department of Biology, TR 48000, Muğla - TURKEY

Received: 24.07.2001

Abstract: The effects of medium and sterilization on the mature embryo culture of barley (*Hordeum vulgare* L.) were studied.

Among the different media used, the highest plantlet development from embryos was obtained in Randolph and Cox medium (92.1%), followed by Murashige and Skoog (MS) (91.3%), half-strength MS (1/2 MS) (87.7%) and B_5 (85.7%).

Among the sterilization methods studied, sodium hypochlorite sterilization followed by treatment of antibiotic solution and sterilization $HgCl_2$ application was found to be more effective than the other methods. However, no statistically significant negative effect of $HgCl_2$ on the plantlet development from embryos was observed.

Key Words: Barley, *Hordeum vulgare*, Embryo culture, Medium, Sterilization.

Arpa (*Hordeum vulgare* L.)'da Embriyo Kültürü

Özet: Arpa (*Hordeum vulgare* L.)'nın olgun embriyolarının kültüründe besin ortamı bileşimi ve sterilizasyon yönteminin etkisi üzerinde durulmuştur.

Kullanılan besin ortamları içerisinde embriyodan bitkicik gelişimi en yüksek Randolph-Cox ortamında elde edilmiştir (%92,1). Bunu sırasıyla Murashige-Skoog (MS) (%91,3); 1/2 MS (%87,7) ve B_5 (%85,7) ortamları izlemiştir.

Araştırılan sterilizasyon yöntemlerinden sodyum hipoklorit ile sterilizasyonu antibiyotik solusyonu ile muamele etme ve $HgCl_2$ ile yapılan sterilizasyon daha etkili bulunmuştur. Ancak, $HgCl_2$'ün embriyodan bitkicik gelişimi üzerine istatistiki bakımdan önemli olmayan olumsuz etkisi de gözlenmiştir.

Anahtar Sözcükler: Arpa, *Hordeum vulgare*, Embriyo kültürü, Besin ortamı, Sterilizasyon.

Introduction

The culturing of an embryo isolated from the seed and ovules of higher plants in special medium is defined as embryo culture. Through embryo culture, either the plant develops directly from the embryo, or first callus formation is stimulated and then shoots and roots occur (indirect organogenesis), so a lot of plants are obtained from just one embryo (1).

Embryo culture is used for various purposes. These include basic studies on embryology, breaking the seed dormancy, testing the vitality of seeds, production of rare species, rescue of embryos that aborted on the mother plant in hybridization interspecific and intergeneric (a great number of hybrid plants have been obtained thus) and production of haploid plants. After the production of haploid plants using embryo culture for the first time in barley, studies increased on the production of haploids in other graminae via this technique (bulbosum technique) (2,3).

The most important aspect of embryo culture is determining a culture medium that will provide the regular growth of embryos that were cultured in different sizes. Nutrients required by embryos vary depending on embryo age. In short, while mature embryos can develop in a simple medium, embryos in the early development stage demand more complex medium (2-6). In various studies, the effects of different solutions on the development of embryos have been investigated

* This work was supported financially by the Research Fund of Muğla University.

and usually MS and B_5 media have been used for the embryo culture of barley (7-10). In this study, MS, 1/2 MS, B_5 and Randolph and Cox media were tested on mature barley embryos.

Furthermore, for successful *in vitro* culture, determining sterilization applications is very important. The sterilization of plant seeds needed for tissue culture can be done with different concentrations of various chemical components (sodium hypochlorite, calcium hypochlorite, mercuric chloride, silver nitrate etc.), fungicides, antibiotics, biocides and temperature applications (11-13). In sterilization procedures, both the concentration and the duration of exposure to disinfectants are important. If the concentration of sterilants is too high or the duration of exposure is too long, the plant tissues will be damaged. If seeds fail to germinate, either the sterilization treatment was too stringent or a lot of seeds were not viable. Since the substances used for sterilization may have a negative effect on germination, sterilization methods are determined through investigations (11,12). The literature should be looked over, the necessary experiments should be conducted and a favorable sterilization method established. Therefore, various sterilization methods with sodium hypochlorite and mercuric chloride were studied.

Materials and Methods

Materials

Plant material: The mature embryos of *Hordeum vulgare* "Kaya 7794" were used. Barley seeds were soaked in sterile distilled water for 24 hours and then embryos were isolated and cultured.

Media: Murashige and Skoog (MS) (1962) basic medium (14), half-strength MS medium (1/2 MS) (14), Randolph and Cox medium (RC) (15) and Gamborg's medium (B_5) (14) were used.

Methods

Studies on Media

MS and 1/2 MS media were supplemented with 30 g/l sucrose and 8 g/l agar, and pH was adjusted to 5.7. B_5 medium is supplemented with 20 g/l sucrose and 8 g/l agar and pH was adjusted to 5.5. These media were prepared according to Bürün and Emiroğlu (1985) (16). Randolph and Cox medium, whose composition is given in Table 1, was prepared similar to other media by drawing 5 ml of each of the two stock solutions (solution A and solution B) into one liter and supplementing with 20 g/l sucrose and 7 g/l agar; pH was adjusted to 5.6 (15).

Seed Sterilization: Twenty-four hours before *in vitro* culture, the seeds were soaked with sterile distilled water. Then they were dipped into 70% ethanol and kept in 4.5% sodium hypochlorite for 5 minutes. Then seeds were rinsed five or six times with sterile distilled water and, after straining the water, the embryos were isolated from seeds.

Culturing of Embryos: The experiment was planned to be repeated twice with 20 culture tubes used in one replication. Two embryos were placed into each culture tube and a total of 40 embryos were cultured in one replication.

The culture tubes were incubated in 16 hour-light and 8 hour-dark photoperiod; the light was 1000 lux. Culture room temperature was about 21-24 °C.

On the 8th day of the culturing process, the rate of plantlet growth from embryo, the length of growing

Table 1. Randolph and Cox medium (15)

Solutions	Concentration of stock solution (g)	The amount to be drawn from stock solution (ml)
Solution A		
$Ca(NO_3)_2$. $4H_2O$	23.6	
KNO_3	8.5	
KCl	56.5	5
Distilled water	500 ml	
Solution B		
$FeSO_4.7H_2O$	0.2	
$(NaPO_3)n$ (Calgon)	1.0	
$MgSO_4$. $7H_2O$	3.6	5
Distilled water	500 ml	

plants, root number and length of the roots were measured and evaluated statistically.

Studies related to the sterilization method

RC medium was used in the optimization of sterilization condition experiments.

Sterilization Method I: Seeds were rinsed in water with a few drops of liquid detergent in a flask for 2-3 minutes and then washed with sterile distilled water 3-4 times. They were dipped in 70% ethanol for 2 minutes and then dipped in 2% sodium hypochlorite with one drop of Tween-80 for 15 minutes and washed with sterile distilled water 5-6 times.

This method of sterilization was studied in two different ways: before and after the seeds are soaked.

Ia. This was the sterilization applied before the seeds were soaked.

After the seeds were sterilized as mentioned above, they were soaked in sterile distilled water for 24 hours and then before culturing they are dipped in 70% ethanol and washed with sterile distilled water.

Ib. This was the sterilization applied as mentioned above after the seeds were soaked.

Sterilization Method II: Seeds were dipped in 70% ethanol. They were kept in 2% sodium hypochlorite for 15 minutes. They were washed with sterile distilled water 5-6 times. They were dipped in antibiotic solution for 30 minutes without being rinsed (antibiotic solution: 600 mg/l penicillin + 250 mg/l streptomycin).

Sterilization Method III: Seeds were dipped for 10 minutes in 0.1% $HgCl_2$ in which one drop of 0.5% HCl was added. They were immersed in 6% hydrogen peroxide (H_2O_2). They were washed with sterile distilled water 3-4 times.

Sterilization Method IV: Seeds were treated with 70% ethanol. They were kept in 4.5% sodium hypochlorite for 5 minutes. They were washed with sterile distilled water 4-5 times.

On the 8th day of the culturing process, contaminations, length of plants, and number and length of roots were measured and evaluated statistically.

Statistical analysis:

Arcsin transformation was performed on the rates of plantlet development from embryos (%) (17). The data were evaluated by random plots design, using the TARİST program (18).

Results and Discussion

Studies on media

The germination of embryos and development of plantlets from embryos were evaluated based on media. In this evaluation, infected embryos were overlooked. Normally both root and shoot development were observed in plantlet development from embryos (Table 2). However, in some embryos, only shoot development without any root development was observed.

Among the media, the highest plantlet regeneration rate was obtained in RC medium (92.1%), followed by in MS (91.3%), 1/2 MS (87.7%) and B_5 (85.7%) media (Table 2).

As a result of statistical analysis, differences among media in terms of plantlet development rates were insignificant.

Many researchers have stated that MS and B_5 media have been used for the culture of mature and immature embryos of barley (7-10,19,20). Also in this study, plantlet development from mature barley embryos in MS and B_5 media was studied. No significant differences were found among media but the best results were obtained in RC (av. 92.1%) and MS (av. 91.3%) media. Gu et al. (1990) determined the effects of various factors affecting immature embryo culture of barley (medium component, size of embryo, genotype etc.) on *in vitro* culture and explained that they obtained better results in MS medium with respect to medium composition (7). In

Table 2. Rates of plantlet development from embryos based on media (%).

Media	Rates of Plantlet Development from Embryos (%)		
	Replication I	Replication II	Average
MS	90.0	92.6	91.3 a
1/2 MS	88.0	87.5	87.7 a
RC	94.4	90.0	92.2 a
B_5	83.3	88.2	85.7 a

the present study, B_5 medium with a rate of 85.7% in terms of plantlet development came in last place. However, there are embryo culture studies claiming that very positive results can be obtained in B_5 medium (10,19,20).

Hill et al. (1992) found that barley embryos growing in *in vitro* culture revealed a 95% germination rate on the 5th day (21). In our study, the highest germination rate was an average of 92.1% in RC medium.

In the present study, average root number, root length and plant length for each embryo growing in different media were determined according to media. Only root number was significant (at 5% level) (Table 3).

Studies related to the sterilization method

On the 12th day of culturing of embryos, their contamination states were observed and, according to sterilization methods, the percentages of contamination rates are given in Table 4.

Between sterilization method Ia (seeds are sterilized before they are soaked) and sterilization method Ib (seeds are sterilised for 24 hours following the soaking), a significant difference in terms of contamination was observed. Sterilization involving covering the seeds in 70% ethanol and then shaking them for 15 minutes in 2% sodium hypochlorite supplemented with one drop of Tween-80 gave better results if applied before the seeds were soaked (contamination 7.8%). When the same method was applied after the seeds were soaked, a 20.2% contamination rate was found (Table 4). Although sterilization method Ia was the second method in which the least contamination was observed, sterilization method Ib was the method in which the highest contamination was observed.

The lowest contamination rate was observed with sterilization method III, which used $HgCl_2$ as sterilant (4.8%).

There was also a focus on whether the sterilization method had an effect on the germination of embryos and plantlet development from embryos. The rates of plantlet development according to sterilization methods are given in Table 4. The highest plantlet development from embryos was observed with sterilization method II. Here as sterilant sodium hypochlorite was used and antibiotic treatment was carried out. In this sterilization method, the rate of contamination was 17.5%, but the rate of plantlet development from embryos was the highest (89.1%).

There are some studies concerning the effects of antibiotic treatment and it is known that antibiotic treatment reduces the rate of infection (22-24). It was reported by Teng and Nicholson (1997) that the treatment of *Panax ginseng* root explants for 40 minutes with 1000 mg/l penicillin-G and 1000 mg/l streptomycin antibiotic solution immediately after sterilization by sodium hypochlorite decreases the rate of contamination considerably; moreover, treatment lasting 2-3 hours lowers the contamination rate to 30-40% (25). However, there are some studies showing that antibiotic solutions are ineffective (26).

Similar to this study, there is another study claiming that the best results in sterilization are obtained with $HgCl_2$ treatment (26). Yet there are some other studies

Table 3. Average root number, root length and plant length for the plantlets growing from embryos.

Media	Root Number	Root Length (cm)	Plant Length (cm)
MS	4.3 a	2.0 a	7.7 a
1/2 MS	4.5 a	1.6 a	8.4 a
RC	3.4 b	1.1 a	6.5 a
B5	3.5 b	1.7 a	6.0 a

Table 4. Contamination rates (%) and plantlet development from embryos according to sterilization methods.

Sterilization Methods	Contamination (%)	Plantlet Development from Embryos (%)
Ia	7.8 a	63.1 bc
Ib	20.2 a	66.2 bc
II	17.5 a	89.1 a
III	4.8 a	81.7 ab
IV	13.1 a	57.8 c

claiming that $HgCl_2$ is harmful to tissues (27). In the present study, the lowest contamination rate was observed in the sterilization applied with $HgCl_2$; however, the rate of plantlet development from embryos was lower than the embryos sterilized with a method supported by antibiotics (81.7%).

The lowest plantlet development was observed with sterilization method IV (57.8%). In this method, commercial bleach (4.5% NaOCl) was applied without being diluted. Although it was applied for a short time (5 minutes), the negative effect of high sodium hypochlorite concentration must be considered.

According to sterilization methods, rates of plantlet development from embryos were found to be significant at 1% level. According to the multiple t test, the best development was observed in culture sterilized with sterilization methods II and III.

As a result, media were listed respectively in terms of plant development as RC, MS, 1/2 MS and B_5. The sterilization with sodium hypochlorite supported with antibiotics and sterilization with $HgCl_2$ were considered to be the best for embryo culture. Embryos were cultured using the first two media and the application of these two sterilization methods, and the following results were obtained (Table 5).

In both media, it was observed that there was an earlier germination and better plantlet development in sterilization method II.

Conclusions

We studied the effects of different media and sterilization methods on embryo culture and no significance differences were found among the studied media. The media were listed best to worst as follows: RC, MS, 1/2 MS and B_5, based on plantlet regeneration from embryos. Randolph and Cox medium was found to be the best medium and we recommend this medium for embryo culture studies since it is simple and can be prepared easily.

Among the sterilization methods, sterilization with sodium hypochlorite followed by treatment of antibiotic (sterilization method II) and sterilization with $HgCl_2$ (sterilization method III) was found more effective. However, in both media (RC and MS) plantlet development from embryos sterilized with $HgCl_2$ was determined to be lower than plantlet development from embryos sterilized with other method (method II).

Table 5. Effect of sterilization with Na-hypochlorite supported with antibiotics or sterilant $HgCl_2$ on the plantlet development in RC and MS media.

Media	Sterilization Method	Number of Embryos Cultured	Infection of Embryos	Contamination (%)	Plantlet Development from Embryos (%)
RC	II	46	2	4.3	95.7
	III	46	-	0	78.3
MS	II	50	1	2	95.9
	III	50	-	0	92.0

References

1. Bürün, B. Buğdaygillerde *in vitro* kültürler. Yüzüncü Yıl Üniv., Ziraat Fakültesi Dergisi, 6 (4): 81-95, 1996.
2. Bürün, B., Gürel, A. Embriyo kültürü. Editörler: M.Babaoğlu, E.Gürel ve S.Özcan, Bitki Biyoteknolojisi I Doku Kültürü ve Uygulamaları, Bölüm 10, ss.324-344, Selçuk Üniversitesi Vakfı Yayınları, Konya, 2001.
3. Monnier, M. Zygotic embryo culture. Editor: S.S. Bhojwani, Plant Tissue Culture: Applications and Limitations, chapter 16, pp. 366-393, Elsevier Science Publishers B.V., The Netherlands, 1990.
4. Monnier, M. Culture of zygotic embryos of higher plants. Eds. J.W. Pollard and J.M. Walker, Plant Cell and Tissue Culture, Methods in Molecular Biology, volume 6, pp.129-139, Humana Press, The Netherlands, 1990.
5. Raghavan, V. *In vitro* methods for the control of fertilization and embryo development, Eds. I.K. Vasil and A. Thorpe, Plant Cell and Tissue Culture, pp.173-194, Kluwer Academic Publishers, The Netherlands, 1994.

6. Hu, C.Y., Zanettini, M.H.B. Embryo culture and embryo rescue for wide cross hybrids, Eds. O.L. Gamborg and G.C. Phillips, Plant Cell, Tissue and Organ Culture, Fundamental Methods, chapter 11, pp.129-141, Springer-Verlag Berlin, Heidelberg, New York, 1995.

7. Gu, D.F., Wu, X.K., Zhang, Y.Z., Zhang, W.Q. Factors affecting callus induction and plantlet regeneration in *in vitro* culture of immature barley embryos. Journal of Jilin Agricultural University, 12:2, 1-6, 107,1990 (CAB Abstracts 1993-1994, ISSN 1000-5684).

8. Oka, S., Saito, N., Kawaguchi, H. Histological observations on initiation and morphogenesis in immature and mature embryo derived callus of barley (*Hordeum vulgare* L.). Annals of Botany, 76: 5, 487-492, 1995.

9. Pan, X.Q., Liang, H.M. Investigation of the medium for barley mature embryo culture. Acta Agronomica Sinica, 17:4, 267-273, 1991 (CAB Abstracts 1993-1994, ISSN 0496-3490).

10. Chung, D.H., Min, K.S., Chun, J.U. Improvement of plant generation rate in *in vitro* cultured haploidy embryos from *H. vulgare* pollinated with *H. bulbosum*. Korean Journal of Crop Science, 39:2, 193-197, 1994 (CAB Abstracts 1995, ISSN 0252-9777).

11. Gamborg, O.L., Phillips, G.C. Sterile Techniques, Eds.O.L. Gamborg and G.C. Phillips, Plant Cell, Tissue and Organ Culture, Fundamental Methods, Chapter 3, p.35-42, Springer-Verlag Berlin, Heidelberg, New York, 1995.

12. George, E.F. Plant Propagation by Tissue Culture, Part 1, The Technology, Chapter 4, Equipment and procedures, pp.95-130, Exegetics Ltd., Edington, Wilts., England, 1993.

13. Babaoğlu, M., Yorgancılar, M., Akbudak, M.A. Doku Kültürü:Temel Laboratuvar Teknikleri, Editörler: M. Babaoğlu, E. Gürel and S. Özcan, Bitki Biyoteknolojisi I Doku Kültürü ve Uygulamaları, Bölüm 1, 1-35, Selçuk Üniversitesi Vakfı Yayınları, 2001.

14. Gamborg, O.L., Phillips, G.C. Media preparation and handling, Eds. O.L. Gamborg and G.C. Phillips, Plant Cell, Tissue and Organ Culture, Fundamental Methods, Chapter 2, p.21-34, Springer-Verlag Berlin, Heidelberg, New York, 1995.

15. Diaz, A. Interspecific hybridization in the genus *Trifolium*. International Training Centre in Genetics and Plant Breeding, Part II, p.499-514, Svalöv-Sweden, 1963.

16. Bürün, B., Emiroğlu, Ü. Besin ortamındaki farklı sükroz konsantrasyonlarının tütünde androgenetik haploidlerin gelişmesine etkisi. Ege Üniversitesi Ziraat Fakültesi Dergisi. 22/2, 29-42, 1985.

17. Açıkgöz, N. Tarımda Araştırma ve Deneme Metodları. Ege Üniv. Ziraat Fak. Yayınları No: 478, Bornova-İzmir, 1988.

18. Anonymous, TARİST, Deneme Değerlendirme Paketi, Seri No:A1001, Ege Üniv. Ziraat Fak. Tarla Bitkileri Bölümü, Bornova, 1999.

19. Rong, J.K., Shu, Q.Y., Hu, S.Q., Li, G.Y. Study on increasing plantlet regeneration rate of barley haploid embryos cultured *in vitro*. Scientia Agricultura Sinica, 24:6, 33-38, 1991 (CAB Abstracts 1993-1994, ISSN 0578-1752).

20. Baek, S.B., Lee, J.H., Kim H.B. Effect of GA_3 concentrations on the vernalization period in the immature embryo culture of barley (*Hordeum vulgare*). Korean Journal of Crop Science, 37:3, 257-263, 1992 (CAB Abstracts 1993-1994, ISSN 0252-9777).

21. Hill, R.D., Durnin, D., Nelson, L.A.K., Abrams, G.D., Gusta, L.V., Abrams, S.R. Effects of (±) -phaseicacid on developing embryos of barley (*Hordeum vulgare* L. cv. Bonanza) cultured *in vitro*. Seed Science Research, 2:4, 207-214, 1992 (CAB Abstracts 1993-1994, ISSN 0960-2585).

22. Poonsapaya, P., Kraisintu, K., Palevitch, D. (Ed), Putievsky, E. Microprogation of *Zingiber cassumunar* Roxb. Acta Horticulturae, No. 344, 557-564, 1993.

23. Chanprame, S., Todd, J.J., Widholm, J.M. Prevention of pink-pigmented methylotrophic bacteria (Methylobacterium mesophilicum) contamination of plant tissue cultures. Plant Cell Reports, 16: 3-4, 222-225, 1996.

24. Salehi, H., Khosh-Khui, M. A simple procedure for disinfection of "Baby Masquerade" miniature rose explants. Scientia Horticulturae, 68:1-4, 145-148, 1997.

25. Teng, W.L., Nicholson, L. Pulse treatments of penicillin-G and streptomycin minimise internal infections and have post-treatment effects on the morphogenesis of ginseng root culture. Plant Cell Reports, 16:8, 531-535, 1997.

26. Seneviratne, P., Flegmann, A.W., Wijesekara, G.A.S. The problem of surface sterilization of shoot materials of Hevea. Journal of the Rubber Research Institute of Sri Lanka, 75: 51-60, 1995 (CAB Abstracts 1995, ISSN 0035-9531).

27. Abou El Dahab, A., Stino, G., El Shiaty, O. Surface sterilization for different parts of the shoot tips of Pritchardia ornamental palms, propagated in vitro. Acta Horticulturae, No. 300, 85-88, 1992.