# Improved regeneration efficiency from mature embryos of barley cultivars

C. AKULA*, A. AKULA and R. HENRY

*Centre for Plant Conservation Genetics, CRC for Molecular Plant Breeding, Southern Cross University Lismore, NSW 2480, Australia*

**Abstract**

A reliable protocol for plant regeneration from mature embryo derived calli of nine barley (*Hordeum vulgare*) cultivars has been developed. The auxins 2,4-dichlorophenoxyacetic acid, picloram and dicamba proved effective in inducing callus from mature embryos of most of the barley cultivars. The induced primary callus was loose, friable and translucent. It ultimately yielded creamy white and compact callus after 2 - 3 transfers on fresh medium of the same composition. Callus induction and regeneration capacity were highly cultivar dependent. Addition of a high concentration of picloram (4 mg dm$^{-3}$) promoted regeneration in 3 cultivars (Tallon, Grimmett and Sloop). In cv. Arapiles, abscisic acid and betaine were crucial in generating morphogenic callus from the mature embryos. Plants regenerated from these calli were hardy and developed roots readily when transferred to hormone free medium.

*Additional key words*: *Hordeum vulgare*, tissue culture, callus induction, genotypes.

## Introduction

Low recovery of green plants from barley callus cultures is a major problem limiting the efficiency of generating transgenic barley. Immature embryos are presently being used as explants for *in vitro* regeneration and have been integrated into a system for recovering transformants (Wan and Lemaux 1994). However, the protocol was based

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*Abbreviations*: ABA - abscisic acid; BAP - 6-benzylaminopurine; CH - casein hydrolysate; 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; KIN - kinetin; NAA - naphthaleneacetic acid.

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Phone: (+61) 266 203407, Fax: (+61) 266 22080, e-mail: dakula@scu.edu.au

*Present address: Institute of Plant Genetic and Crop Plant Research, Correnstrasse 3, D-06466 Gatersleben, Germany

on the model barley, Golden Promise and has had poor success when applied to other cultivars (Bregitzer *et al.* 1998). Mature dry seeds may be considered for this purpose using the mature embryos as explants.

The use of mature embryos from dry seed has several advantages; mature tissue is easy to handle, available year round and in bulk quantities. *In vitro* induction of shoot buds from the calli derived from mature embryos was more successful in pearl millet (Botti and Vasil 1983), wheat (Heyser *et al.* 1985, Ozgen *et al.* 1998), oat (Torbert *et al.* 1998) and rice (Rueb *et al.* 1994; Khanna and Raina 1997) than in barley. Bayliss nd Dunn (1979) reported induction of callus from mature embryos of barley. Lupotto 1984) was able to regenerate 2 green shoots from a single cultivar, Maxima. Rengel (1987) reported regeneration of plants via somatic embryogenesis from the callus cultures derived from mature embryos of 8 lines of barley. Taniguchi *et al.* (1991) successfully regenerated shoot buds from mature embryo derived calli only in 14 out of 203 cultivars tested, but did not provide protocols for regeneration. The aim of this research was to find a highly reproducible regeneration system from the excised mature embryos of dry seeds of a range of Australian malting barley cultivars.

## Materials and methods

Mature dry seeds of *Hordeum vulgare* L. (cultivars: Clipper, Stirling, Arapiles, Franklin, Schooner, Lindwall, Tallon, Grimmett, Sloop) were kindly supplied by Hermitage Research Station, Warwick, UK. Seeds were sterilised by 70 % ethanol, fungicide (benomyl) and 0.1 % mercuric chloride and soaked in sterile double distilled water for 2 h. The embryos were excised from the seeds and the meristematic zones (plumula and coleorhiza) were slightly damaged to inhibit the normal germination. Eight embryos with scutellar tissue in contact with the medium were incubated in Petri dishes containing nutrient agar medium. Each experiment represents 4 plates with 8 excised embryos in each dish. The basal media used throughout this study were either MS (Murashige and Skoog 1962) or N6 (Chu 1978), solidified with 0.8 % agar (*Sigma*) with different supplements:

R-1 medium: N6 basal salts with no organic supplements + 3 % sucrose

R-2 medium: N6 salts + 5 mg $dm^{-3}$ thymine HCl + 5 mg $dm^{-3}$ pyridoxine + 5 mg $dm^{-3}$ nicotinic acid + 500 mg $dm^{-3}$ myo-inositol + 1 g $dm^{-3}$ casein hydrolysate + 3 % sucrose

R-3 medium: MS salts + vitamines + 0.6 % maltose + 2 % sorbitol

R-4 medium: 1/2 strength MS salts with full strength vitamines + 0.6 % maltose + 2 % sorbitol

R-5 medium: MS salts with reduced ammonium nitrate (0. 165 g $dm^{-3}$) + 4 mg $dm^{-3}$ thymine HCl + 100 mg $dm^{-3}$ myo-inositol + 730 mg $dm^{-3}$ glutamine + 0.6 % maltose.

All hormones and other growth factors (except picloram and dicamba) including vitamins were added to the medium and the pH adjusted to 5.75 before autoclaving. All the media were autoclaved at 115 kPa, 120 °C for 20 min. Dicamba and picloram were filter sterilised before adding to the autoclaved medium. All the cultures were maintained under cool white fluorescent lamps providing approximately 30 - 40 μmol(photon) $m^{-2} s^{-1}$ over a 16-h photoperiod, at temperature 24 - 26 °C. Adventitious shoots with 3 - 5 leaves regenerated from the calli were transferred to BCI medium without growth regulator as described by Tingay *et al.* (1997) which encouraged root proliferation and normal growth and development of plantlets.

## Results

**Induction of primary callus:** All the cultivars tested were able to induce loose, soft and translucent calli (described as type A) from the excised mature embryos within 3 - 4 weeks of incubation on the induction medium (R-2) (Table 1). 80 - 95 % responses could be achieved by careful selection of uniform, healthy and viable seeds. All the cultivars except Lindwall initially induced loose, granular and watery callus (type A). Surprisingly we did not find any significant change in the callus quantity or quality (except Arapiles) between the two concentrations of auxins tested. However, in cultivar Arapiles, 2 mg $dm^{-3}$ of 2,4-D yielded more compact and white calli (B type) at a slower rate, whereas by doubling the auxin concentration the mature embryos yielded more loose and soft callus (type A). Lindwall also showed type B callus. The callus quality of Arapiles varied greatly with different growth regulators in the medium. ABA and betaine induced a unique type of callus by producing yellowish white and compact callus in Arapiles but failed to induce callus with other cultivars.

Table 1. Cultivar differences on the ability of callus induction of excised mature embryos of barley on R-2 medium supplemented with 2 or 4 mg $dm^{-3}$ 2,4-D, 2.5 or 4 mg $dm^{-3}$ dicamba, and 2 or 4 mg $dm^{-3}$ picloram. Callus type A - loose, translucent and soft; callus type B - creamy white and compact. NR- no response; ++ - < 200 mg, +++ - 200 - 500 mg, ++++ - 500 - 600 mg fresh mass of callus obtained.

| Cultivar | 2,4 D | | Dicamba | | Picloram | |
|---|---|---|---|---|---|---|
| Clipper | NR | | ++ | A, 70 % | NR | |
| Stirling | NR | | ++ | A, 50 % | NR | |
| Tallon | NR | | ++ | A, 80 % | ++++ | A, 70 % |
| Arapiles | ++ | A, 50 % | NR | | NR | |
| Franklin | ++ | A, 70 % | ++ | A, 50 % | NR | |
| Schooner | ++ | A, 30 % | NR | | +++ | A, 80 % |
| Lindwall | ++ | B, 60 % | ++ | A, 80 % | ++++ | A, 70 % |
| Grimmet | ++ | A, 32 % | ++ | A, 75 % | +++ | A. 80 % |
| Sloop | +++ | A, 76 % | ++ | A, 93 % | +++ | A, 83 % |

No callus formation was observed when betaine was replaced with L-proline. Neither low nor high concentrations of dicamba and picloram were able to induce callus in Arapiles. Arapiles responded very specifically to 2,4-D in callus induction. Lindwall also showed type B callus with 2,4-D. Sloop also responded to all types of auxin. Picloram was found to be the best choice of auxin in generating green and compact callus. Clipper and Stirling were very specific in requiring dicamba for callus induction. Neither picloram nor 2,4-D able to induce callus from mature embryos of these cultivars. IBA and BAP did not produce any signs of callus formation at any stage/age of culture, instead, they promoted normal germination of the excised embryos.

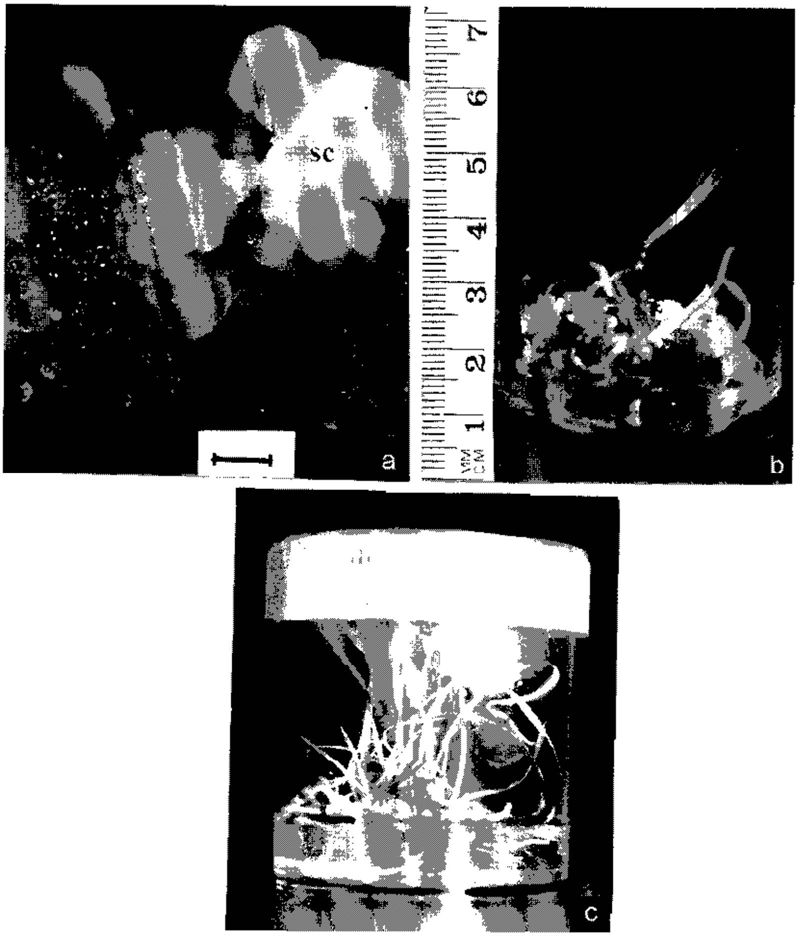


Fig. 1. Regeneration of barley cv. Sloop. *a* - 11-week-old calli initiated from the excised mature embryos on R-2 medium supplemented with 2 mg dm$^{-3}$ picloram. The primary callus (pc) is loose and friable of type A, which produced compact creamy whitish secondary callus (sc) of type B (*bar* = 1 mm). *b* - secondary calli yielded more than 7 well developed leafy shoots within 3 - 4 weeks of incubation on R-2 medium with 1 mg dm$^{-3}$ 2,4 D and 1 mg dm$^{-3}$ KIN. *c* - advanced stage of plantlet showing profuse rooting after 3 weeks of incubation on BCI medium.

**Formation of secondary callus:** In general, during multiplication of this calli by transferring to fresh medium of the same composition, we observed very significant qualitative changes. The phenotypic appearance varied from cultivar to cultivar. In the case of Clipper and Stirling, the callus mass did not show any signs of further multiplication, instead, they showed initiation of greenish adventitious roots. The initial callus formed from the mature embryos of Franklin and Arapiles turned brownish black and degenerated after 2 subsequent culture transfers. In only four cultivars out of the 9 tested, Grimmett, Tallon, Sloop and Lindwall the initial callus type of A is changed to milky white, compact and nodular type of calli (type B) formed on the surface of the original calli (Fig. 1*a*). Secondary calli thus formed was subdivided and transferred to fresh medium of the same composition to encourage further development and proliferation. In this way we could able to generate 2 - 2.5 g of fresh mass of type B callus from the original individual callus mass within 8 - 10 week period. However, the original cultures if remained in the same medium without sub-culturing also produced secondary calli of type B, but at slow rate.

Table 2. Morphogenic changes in the secondary calli and percentage of calli responding of different barley cultivars challenged on culture media R-1 to R-5 with different supplements [mg $dm^{-3}$]. Observations made from 3-week-old cultures.

| Medium | Tallon | Sloop | Grimmett | Lindwall |
|---|---|---|---|---|
| R-1 | roots, 80 % | no change | no change | degeneration |
| R-2 | roots, 80 % | no change | no change | degeneration |
| R-2 + 1 2,4-D + 1 KIN | roots, 90 % | shoots, 40 % | roots, 60 % | roots, 60 % |
| R-2 + 0.8 NAA + 1 KIN | roots, 10 % | shoots, 19 % | roots, 29 % | roots, 35 % |
| R-2 + 0.8 NAA + 2 KIN | roots, 10 % | shoots, 46 % | roots, 23 % | roots, 35 % |
| R-2 + 0.05 BAP | no change | no change | no change | no change |
| R-2 + 0.1 BAP + 0.15 KIN | brown callus | no change | no change | no change |
| R-2 + 2 2,4-D + 80 proline | loose callus | buds, 50 % | no change | plantlets, 45 % |
| R-3 + 0.5 2,4-D + 1 KIN + 60 000 maltose + 20 000 sorbitol | greenish callus, 48 % | roots, 60 % | friable callus | plantlets, 50 % |
| R-4 + 0.5 2,4-D + 1 KIN | greenish callus | roots, 60 % | friable callus | plantlets, 50 % |
| R-5 + 2 BAP | roots, 92 % | roots, 85 % | roots, 75 % | no change |
| R-3 + 5 thymine + 1 000 myo-inositol + 0.5 KIN + 500 CH + 30 000 sucrose | embryoid | roots, 73 % | roots, 86 % | roots, 67 % |

**Comparison of the regeneration of different cultivars:** Seven-week old secondary calli of Tallon, Sloop, Grimmett and Lindwall was challenged on 12 different media (Table 2) to test regeneration potential. We succeeded in inducing shoot bud formation from secondary calli in Sloop and Lindwall. However, none of these media resulted in morphogenic response from the secondary callus of Grimmett. Hormone free basal media either with or without organic supplements failed to produce any signs of morphogenic stimulus in Sloop, Grimmett and Lindwall, but in Tallon profuse root formation was observed. Sloop responded within 3 weeks of incubation on R-2 medium supplemented with 2,4-D and KIN by giving out leafy shoots (Fig. 1*b*). But the calli of the other 3 cultivars induced roots but failed to initiate adventitious shoots. The regeneration media containing NAA promoted root formation in all the cultivars tested. In case of the calli of Sloop, the effect of NAA was balanced by doubling the concentration of KIN, producing leafy shoots that failed to develop further. Low concentrations of BAP (0.05 and 0.1 mg $dm^{-3}$) in the regeneration medium did not show any signs of morphogenic impact. Addition of proline (80 mg $dm^{-3}$) or sorbitol (20 g $dm^{-3}$) promoted shoot bud initiation from the calli of Lindwall and yielded 2 - 5 shoots from each individual callus. Sorbitol also favoured induction of somatic embryoids on the callus surface in Tallon, but they failed to transform into plantlets. Low frequency shoot formation also occurred in 60 % of calli of Sloop when the medium was supplemented with 2 mg $dm^{-3}$ 2,4-D and 80 mg $dm^{-3}$ L-proline. Wide variations in culture response were noticed both within and between cultivars. Sloop and Lindwall were able to induce regeneration in more than one medium out of 12 combinations tested. Tallon was close to developing green shoots, whereas the calli of Grimmett showed more morphogenic tendency towards root formation.

**Optimising regeneration media for each cultivar:** In separate experiments, excised mature embryos of Tallon, Grimmett and Sloop were directly incubated on R-2 medium supplemented with 1 mg $dm^{-3}$ 2,4-D and 2 mg $dm^{-3}$ KIN. Observations from 3-week-old cultures indicated that mature embryos of Sloop initiated loose and translucent callus from the entire surface of the embryo at a rapid rate. But the process was slower in Grimmett and Tallon (Table 3). The response in these two cultivars was relatively low (below 50 %) compared to Sloop (80 %).

Table 3. Callus induction and plant regeneration from excised mature embryos in 3 cultivars.

| Medium [mg $dm^{-3}$] | Callus induction<br>R-2 + 1 2,4-D + 2 KIN | Secondary callus<br>R-3 + 2 2,4-D + 2 KIN | Shoot formation<br>R-2 + 4 picloram |
|---|---|---|---|
| Sloop | rapid, A, +++, 80 % | creamy white, 60 % | 5 - 7 shoots, 60 % |
| Grimmett | slow, A, ++, 49 % | creamy white, 52 % | 3 - 7 shoots, 57 % |
| Tallon | slow, A, ++, 48 % | creamy white, 80 % | 4 - 7 shoots, 49 % |

Uniformly responding 5-week-old cultures with primary callus were selected and transferred to fresh medium after doubling the concentration of 2,4-D. More than 60 % of the calli responded by producing creamy white and compact secondary calli of type B within 4 weeks of incubation. This calli were challenged for morphogenic response on R-2 medium supplemented with a high concentration of picloram. Picloram induced shoot regeneration from the secondary calli within 2 - 4 weeks of incubation in all 3 cultivars. The highest number of cultures of Sloop (60 %) showed shoot formation followed by Grimmett (57 %) and Tallon (49 %). The secondary calli of Sloop cultures also yielded the highest number of plants (5 - 7) from a single culture.

Six-week-old yellowish compact callus (induced by R-2 medium supplemented with ABA and betaine) derived from the excised mature embryos of Arapiles was challenged on the same medium (R-2) by replacing ABA and betaine with 0.1 mg $dm^{-3}$ BAP and 0.15 mg $dm^{-3}$ KIN. After a 4 week period of incubation 10 % of the cultures yielded 2 - 5 green leafy shoots from a single callus clump. After

6 weeks of incubation 50 % of the cultures regenerated shoot buds with or without simultaneous root formation.

**Development of complete plantlets:** Individual leafy shoots with 3 - 5 leaves were separated from each other from the clumps of the calli and transferred to BCI medium as described by Tingay *et al.* (1997) without growth regulators for normal development. Profuse root formation was observed with in 2 - 3 weeks of incubation (Fig. 1c). All the plantlets were green, hardy and healthy. No albinos were observed at any stage throughout the experiment.

## Discussion

In cereals, the most readily available donor material for tissue culture is the mature embryo. However, mature embryos are considered more recalcitrant to tissue culture than immature embryos. However, because of the problems linked with immature embryos, the focus is now shifted towards mature embryos. A highly efficient regeneration and transformation protocol has been recently developed in rice (Abedinia *et al.* 1997) and oat (Tolbert *et al.* 1998) which stimulated our interest in extending this approach to barley mature embryos. In barley, genotypic restrictions impended the use of the protocols based on the results of previous investigators (Lupotto 1984, Rengel 1987, Taniguchi *et al.* 1991). Attempts to reproduce these methods with our cultivars yielded negative results (data not presented). The current work demonstrated greater success with five commercial cultivars (Lindwall, Tallon, Sloop, Grimmett and Arapiles) based on diverse media formulations and culture conditions, which confirmed the genotypic dependence of the regeneration technique. In the present investigation, cultivar variability was not only for callus formation but also in morphogenic responses and regeneration ability. Such variations in barley have been documented both with mature embryos (Taniguchi *et al.* 1991, Rengel 1987) and also with immature embryos (Bregitzer *et al.* 1998). After extensive investigation with a wide range of auxins, we found 3 synthetic auxins 2,4-D, dicamba and picloram suitable for callus induction from mature embryos of most of the barley genotypes. Out of the wide range of media compositions we tested, we found only 12 media combinations, on which, it was possible to induce formation of green shoots (the highest number of shoots was 7 from a single calli). Initial callus of type A and subsequent change to type B was also reported earlier from mature embryos (Lupotto 1984, Rengel 1987, Taniguchi *et al.* 1991) and also from shoot tips (Weigel and Hughes 1985) of barley.

Our study clearly showed that auxins inducing callus formation were cultivar specific, *e.g.*, dicamba was suitable for Clipper and Stirling and 2,4-D for Lindwall, Grimmett and Sloop. On the other hand, in Arapiles, ABA and betaine induced regenerable callus. Beneficial effects of ABA in obtaining regeneration in monocots was reported earlier in wheat (Brown *et al.* 1989), rice (Suprasanna *et al.* 1997) and in pearl millet (Vasil and Vasil 1981, 1982). In barley, Rengel (1986) obtained a large number of green plantlets via somatic embryos from callus cultures incubated on MS medium supplemented with ABA. Stress mediated ABA synthesis is known to induce a morphogenic response in plant cultures (Brown *et al.* 1989).

We established a common protocol suitable to 3 genotypes with a regeneration efficiency ranging from 3 - 7 plants per single callus. An important observation was that a high concentration (4 mg $dm^{-3}$) of picloram induced the initiation of adventitious shoots from callus cultures of Tallon, Lindwall and Grimmett. Kachhwaha (1997) also reported the use of picloram during the induction of somatic embryos from immature embryo derived calli in barley. Further, specific cases of auxin dependency during regeneration was also reported in finger millet (Eapen and George 1989), sugarcane (Nadar *et al.* 1978) and pearl millet (Haydu and Vasil 1981).

Barley mature embryo regeneration is proved to be genotype specific. These results are in aggreement with those of Rengel (1987) who suggested that all genotypes in barley have the ability to form callus and to regenerate, but in various ways. This is also true with the explant type. The induction of regeneration from any explant can be achieved by various cultural manipulations. We succeeded inducing shoot buds in reasonable numbers from the mature embryo derived calli in five commercial cultivars out of the 9 tested. Further studies to optimise the sources of nitrogen and carbon and culture regimes at different stages of culture development, are presently under way. This will enhance the regeneration efficiency for successful implementation in transformation protocols.

**References**

Abedinia, M., Henry, R.J., Blakeney, A.B., Lewin, L.: An efficient transformation system for the Australian rice cultivar, Jarrah. - Aust. J. Plant Physiol. **24**: 133-141, 1997.

Bayliss, M.W., Dunn, S.D.M.: Factors affecting callus formation from embryos of barley (*Hordeum vulgare*). - Plant. Sci. **14**: 311-316, 1979.

Botti, C., Vasil, I.K.: Plant regeneration by somatic embryogenesis from parts of cultured mature embryos of *Pennisetum americanum* (L.) Schum. - Z. Pflanzenphysiol. **111**: 319-325, 1983.

Bregitzer, P., Dahleen, L.S., Campbell, R.D.: Enhancement of plant regeneration from callus of commercial barley cultivars. - Plant Cell Rep. **17**: 941-945, 1998.

Brown, C., Brooks, F.J., Pearson, D., Mathias, R.J.: Control of embryogenesis and organogensis in immature wheat embryo callus using increased osmolarity and abscisic acid. - J. Plant Physiol. **133**: 727-733, 1989.

Chu, C.C.: The N6 medium and its application to anther culture of cereal crops. - In: Proceedings of the Symposium Plant Tissue Culture. Pp. 45-50. Science Press, Peking 1978.

Eapen, S., George, L.: High frequency plant regeneration through somatic embryogensis in finger millet (*Eleusine coracana* (L.) Gaertn.). - Plant Sci. **61**: 127-130, 1989.

Haydu, Z., Vasil, I.K.: Somatic embryogenesis and plant regeneration from leaf tissues and anthers of *Pennisetum purpureum* Schum. - Theor. appl. Genet. **59**: 269-273, 1981.

Heyser, J.W., Nabors, M.W., Mackinnon, C., Dykes, T.A., DeMott, K.J., Kautzmann, D.C., Mujeeb-Kazi, A.: Long-term, high-frequency plant regeneration and the induction of somatic embryogenesis in callus cultures of wheat (*Triticum aestivum* L.). - Z. Pflanzenzücht. **94**: 218-233, 1985.

Kachhwaha, S., Varshney, A. Kothari, S.L. Somatic embryogenesis and long term high plant regeneration from barley (*Hordeum vulgare*). - Cereal Res. Commun. **25**: 117-124, 1997.

Khanna, H., Raina, S.K.: Enhanced *in vitro* plantlet regeneration from mature embryo-derived primary callus of a Basmati rice cultivar through modification of nitrate-nitrogen and ammonium-nitrogen concentrations. - J. Plant Biochem. Biotechnol. **6**: 85-89, 1997.

Lupotto, E.: Callus induction and plant regeneration from barley mature embryos. Ann. Bot. **54**: 523-529, 1984.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Plant Physiol. **15**: 473-497, 1962.

Nadar, H.M., Soepraptopo, S., Heinz, D.J., Ladd, S.L.: Fine structure of sugarcane (*Saccharum* sp.) callus and the role of auxin in embryogenesis. - Crop Sci. **18**: 210-216, 1978.

Ozgen, M., Turet, M., Altınok, S., Sancak, C.: Efficient callus induction and plant regeneration from mature embryo culture of winter wheat (*Triticum aestivum* L.) genotypes. - Plant Cell Rep. **18**: 331-335, 1998.

Rengel, Z.: Embryogenic callus induction and plant regeneration from cultured *Hordeum vulgare* mature embryos. - Plant Physiol. Biochem. **25**: 43-48, 1987.

Rengel, Z.: Effect of abscisic acid on plant development from *Hordeum vulgare* embryogenic callus. - Biochem. Physiol. Pflanz. **181**: 605-610, 1986.

Rueb, S.M., Leneman, M., Schilperoort, R.A., Hensgens, L.A.M.: Efficient plant regeneration through somatic embryogenesis from callus induced on mature rice embryos (*Oryza sativa*). - Plant Cell Tissue Organ Cult. **36**: 259-264, 1994.

Suprasanna, P., Ganapathi, T.R., Rao, P.S.: Embryogenic ability in long term callus cultures of rice (*Oryza sativa*). - Cereal Res. Commun. **25**: 27-33, 1997.

Taniguchi, M., Enomoto, S., Komatsuda.: Varietal difference in the ability of callus formation and plant regeneration from mature embryo in barley (*Hordeum vulgare*). - Jap. J. Breed. **41**: 571-579, 1991.

Tingay, S.D., McElroy, D., Kalla, R., Fieg, S., Wang, M., Thornton, S., Brettell, R.: *Agrobacterium tumefaciens*-mediated barley transformation. - Plant J. **11**: 1369-1376, 1997.

Torbert, K. A., Rines, H.W., Somers, D.A.: Transformation of oat using mature embryo derived tissue cultures. - Crop Sci. **38**: 226-231, 1998.

Vasil, V., Vasil, I.K.: Somatic embryogenesis and plant regeneration from suspension cultures of pearl millet (*Pennisetum americanum*). - Ann. Bot. **47**: 669-678, 1981.

Vasil, V., Vasil, I.K.: Characterisation of embryogenic cell suspension cultures derived from cultured inflorescences of *Pennisetum americanum*. - Amer. J. Bot. **69**: 1441-1449, 1982.

Wan, Y., Lemaux, P.G.: Generation of large numbers of independently transformed fertile barley plants. - Plant Physiol. **104**: 37-48, 1994.

Weigel, R.C., Hughes, K.W.: Long-term regeneration by somatic embryogenesis in barley (*Hordeum vulgare*) tissue cultures derived from apical meristem explants. - Plant Cell Tissue Organ Cult. **5**: 151-162, 1985.