BIOLOGIA PLANTARUM 48 (3): 473-476, 2004

BRIEF COMMUNICATION

Callus induction and *in vitro* regeneration from barley mature embryos

J.M. ZAPATA, B. SABATER and M. MARTÍN*

Departamento de Biología Vegetal, Universidad de Alcalá, Alcalá de Henares, E- 28871 Madrid, Spain

Abstract

We have assayed different combinations of nutrient media and growth regulators to induce callus and plant regeneration from explants of root, shoot and leaf, complete seed, and isolated mature embryo of barley (*Hordeum vulgare* L. cv. Hassan). The best results were obtained with mature embryo in J25-8 medium supplemented with 2.0 mg dm^{-3} 2,4-dichlorophenoxyacetic acid where about 75 % developed friable calli. Some 80 - 85 % of these calli regenerated barley plants in the same J25-8 medium supplemented with 1.0 mg dm^{-3} indole-3-butyric acid and 0.1 mg dm^{-3} kinetin.

Additional key words: 2,4-D, *Hordeum vulgare*, IBA, kinetin, NAA, organogenesis.

A large number of plant species (monocotyledonous and dicotyledonous) have been introduced into tissue culture. Callus cultures capable of somatic embryogenesis are possible in graminaceous plants when the explants employed are "embryonic" in nature (Vikrant and Rashid 2003). Besides an embryonic explant, another prerequisite for the initiation of an embryogenic callus culture is relatively high concentration of a synthetic auxin such as 2,4-D (Vikrant and Rashid 2003). Tissue culture has several potential uses in barley and is an important part of successful transformation procedures (Wan and Lemaux 1994). However, one current limitation in barley transformation is the poor regeneration of modern cultivars (Bregitzer *et al.* 1998) and most of graminaceous crops have problems in establishing regenerable tissue cultures (Koprek *et al.* 1996).

Callus derived from immature embryo was successfully employed to yield regenerated plants (Bregitzer *et al.* 1998). However, regeneration from embryo-derived callus from barley is controlled by several genes (Komatsuda *et al.* 1989, Mano *et al.* 1996, Bregitzer *et al.* 1998) and there is a large variability of responses among different genotypes (Bregitzer 1992, Baillie *et al.* 1993). Mature embryo has not been investigated in detail because of the difficulty to obtain tissue cultures with high rates of plant regeneration. The aim of the present work was to optimize the culture conditions (medium, growth regulators) for callus induction and regeneration of plants from different explants of barley.

We have assayed the induction of callus from complete seed, isolated embryo, and explants of root, shoot and leaf of 7 and 14-d-old barley (*Hordeum vulgare* L. cv. Hassan) grown on vermiculite in a growth chamber (Martín *et al.* 1986). For each culture condition, 200 explants, 100 complete seeds (embryo with endosperm) of no more than 3-year-old and 100 mature embryos (from seeds previously sterilized and cut through the root-shoot axis under sterile conditions to remove endosperm) were sown.

Explants and seeds were washed in distilled water, and then shaken in a 20 % (v/v) aqueous solution of *Domestos*® (*Lever Bros*, London, UK) for 20 and 30 min, respectively. After three washes in sterile water, explants, seeds and mature embryos were sown on the solid culture media: MS (Murashige and Skoog 1962), B_5 (Gamborg *et al.* 1968), N_6 (Chu 1978), SH (Schenk and Hildebrand 1972) and J25-8 (Jensen 1977). Unless otherwise noted, the media were supplemented with 250 mg dm^{-3} casein hydrolysate, 30 g dm^{-3} sucrose and 0.8 % (m/v) agar. The pH was adjusted to 5.8. Cultures were maintained

Received 12 May 2003, *accepted* 21 March 2004.
Abbreviations: 2,4-D - 2,4-dichlorophenoxyacetic acid; IBA - indole-3-butyric acid; KIN - kinetin; NAA - α-naphtalene acetic acid.
Acknowledgements: This work was supported by a Grant from the Spanish DGICYT (BFI2000-0780).
* Corresponding author; fax: (+34) 91 885 5066, e-mail: mercedes.martin@uah.es

at 25 °C under a 16-h photoperiod with irradiance of 20 W m^{-2} (white light) or in the dark. We have studied the morphogenetic effects on these explants of different combinations of growth regulators: α-naphtalene acetic acid (NAA) and kinetin (KIN) (1.0 and 2.0 mg dm^{-3} for each one), and 2,4-dichloro-phenoxyacetic acid (2,4-D) (1.0, 2.0 and 3.0 mg dm^{-3}). In a few cases, the concentration of growth regulators in the medium was 0.5 mg dm^{-3}.

No significant differences on callus induction were found between light and dark incubation (data not shown). Table 1 shows the percentages of callus induction of cultures in the dark only for those growth regulator applications that induced callus in at least one plant material. N_6 and SH media did not induce callus in any of the material assayed. No culture conditions induced callus in leaf explants that became chlorotic 48 h after sowing.

The MS medium supplemented with 2.0 mg dm^{-3} 2,4-D allowed the induction of callus from root (28.5 %), shoot (23 %), seed (27 %) and mature embryo (35 %). When MS medium was supplemented with NAA (1.0 mg dm^{-3}) and KIN (2.0 mg dm^{-3}), callus was only induced from root (18 %) and seed (20%). Although MS medium with appropriate growth regulators was very efficient in callus induction during the first two weeks of

Table 1. Effects of 2,4-D and NAA + KIN on callus induction from root, shoot, seed and mature embryo of barley on MS, B_5 and J25-8 media. The cultures were grown in dark. Indicated values are percentages of callus induced in respect of the number of samples sowed (200 for explants and 100 for seeds and mature embryos).

Medium	Growth regulators [mg dm^{-3}]	Root [%]	Shoot [%]	Seed [%]	Embryo [%]
MS	2 2,4-D	28.5	23	27	35
	1 NAA + 2 KIN	18	0	20	0
B_5	2 2,4-D	0	0	19	0
	3 2,4-D	12.5	0	0	0
J25-8	2 2,4-D	35	0	37	75

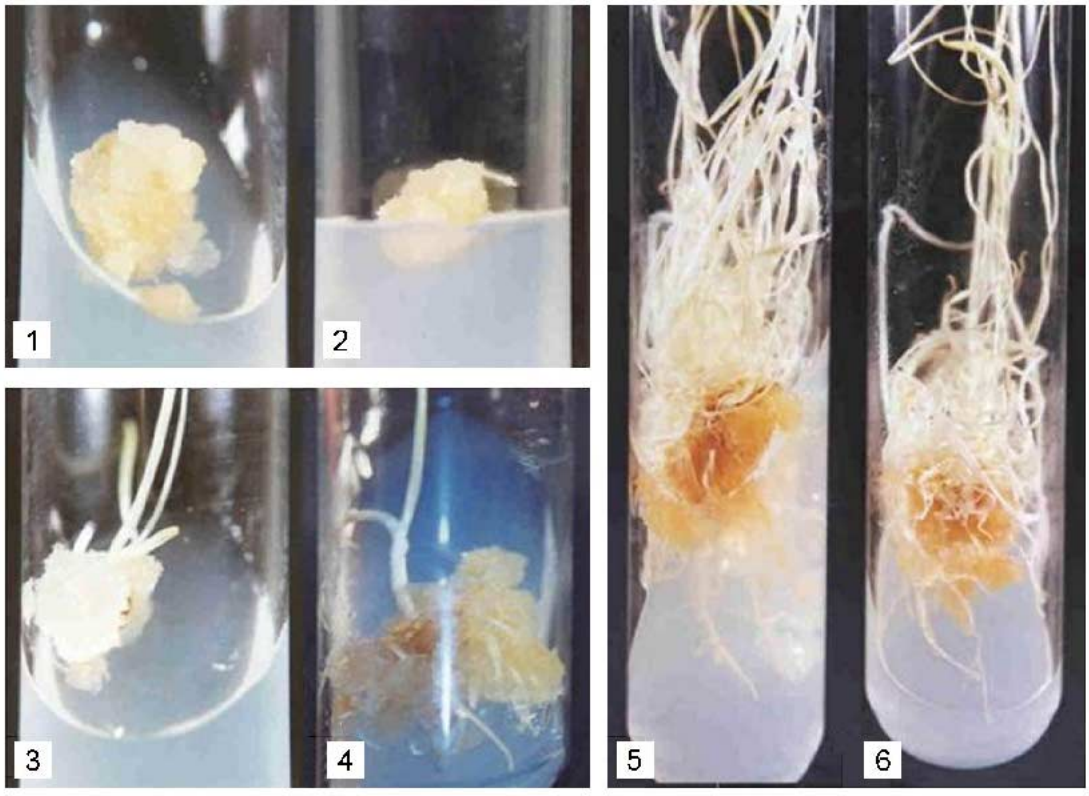

Fig. 1. Establishment of callus and regeneration of barley plants from mature embryos in the dark (1: eight-day callus, 2 - 6: regenerated plants after 4, 6, 7, 9 and 10 weeks, respectively).

culture, growth did not continue for more than four weeks. On the other hand, B_5 medium supplemented with 3 and 2 mg dm^{-3} of 2,4-D was efficient to induce microcallus from root (12.5 %) and seed (19 %), respectively (Table 1), but these cultures did not grow more than one week. The highest percentage of callus induction was obtained with J25-8 medium supplemented with 2.0 mg dm^{-3} 2,4-D. In these conditions, roots (35 %) and complete seeds (37 %) were able to produce callus. However, these calli grew slowly, they have barely duplicated their masses after two months. When mature embryos were placed on the J25-8 medium supplemented with 2.0 mg dm^{-3} 2,4-D callus induction was 75 % after 10 d of culture (Table 1). These conditions induced rapid and green friable callus at white light and albino friable callus in the dark. 3.0 and 4.0 mg dm^{-3} 2,4-D further increase callus induction and growth (results not shown). However, to reduce the negative effect of high concentrations of 2,4-D on plant regeneration (Gamborg *et al*. 1968), we worked with 2.0 mg dm^{-3} 2,4-D. Dark and light grown calli were regularly subcultured after 4 weeks for at least 2 years.

To regenerate barley plants from callus obtained from mature embryo, we assayed different combinations of growth regulators. The highest percentage of regeneration was obtained with IBA + KIN (1:10) supplemented to J25-8 medium. Friable calli (4-week subcultured up to 2 years) were placed on J25-8 supplemented with 1.0 mg dm^{-3} IBA and 0.1 mg dm^{-3} KIN at 25 °C under a 16-h photoperiod (20 W m^{-2}; white light) or in the dark. After one week rhizogenesis was detected in both albino and green plants. Three weeks later, green or albino vigorously growing plants were regenerated (Fig. 1). About 85 and 80 % calli (of about 80 cultured in each condition) regenerated albino and green plants in the dark and at light, respectively. All regenerated plantlets appeared to be phenotypically normal.

Specific modifications of the culture medium strongly influence the plant regeneration from embryogenic barley callus, which in various cultivars depends on 2,4-D concentration (Bregitzer *et al.* 1998) although 3.0 mg dm^{-3} is adequate for most of them. Our results also showed the requirement of 2.0 mg dm^{-3} 2,4-D for the establishment of embryogenic calli (Vikrant and Rashid 2003). In addition, we found that culture medium is also critical for callus induction and plant regeneration in the cv. Hassan. Therefore, the N_6 medium, that was suitable for cereal anther culture (Chu 1978) and immature embryos from maize (Zacchini *et al.* 2003), did not induce callus in the barley cv. Hassan. Similarly the SH medium, that was introduced for the induction and culture of callus of both monocotyledonous and dicotyledonous plants (George 1993), was not efficient for the barley. Gamborg *et al.* (1968) found that the regenerative ability of barley cell suspensions declined with the time in B_5 containing 2,4-D. In this line, we found that B_5 supplemented with different concentration of 2,4-D was unable to support continuous calli growth.

Our results demonstrated that the J25-8 medium supplemented with 2.0 mg dm^{-3} of 2,4-D is highly efficient for callus growth and plant regeneration (85 % of regenerated plants).

References

Baillie, A.M.R., Rossnagel, B.G., Kartha, K.K.: Evaluation of 10 Canadian barley (*Hordeum vulgare* L.) cultivars for tissue culture response. - Can. J. Plant Sci. **73**: 171-174, 1993.

Bregitzer, P.: Plant regeneration and callus type in barley: Effects of genotype and culture medium. - Crop Sci **32**: 1108-1112, 1992.

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.

Chu, C.C.: The N_6 medium and its applications to anther culture of cereal crops. - In: Proceedings of Symposium of Plant Tissue Culture. Pp. 45-50. Science Press, Peking 1978.

Gamborg, O.L., Miller, R.A., Ojima, K.: Nutrient requirements of suspension cultures of soybean root cells. - Exp. Cell Res. **50**: 151-158, 1968.

George, E.F.: Plant Propagation by Tissue Culture. - Exegetics, Ltd., Edington 1993.

Jensen, C.J.: Monoploid production by chromosome elimination. - In: Reinert, J., Bajaj, Y.P.S. (ed.): Applied and Fundamental Aspects of Plant Cell, Tissue and Organ Culture. Pp. 299-330. Springer-Verlag, Berlin 1977.

Komatsuda, T., Enomoto, S., Nakajima, K.: Genetics of callus proliferation and shoot differentiation in barley. - J. Hered. **80**: 345-350, 1989.

Koprek, T., Hänsch, R., Nerlich, A., Mendel, R.R., Schulze, J.: Fertile transgenic barley of different cultivars obtained by adjustment of bombardment conditions to tissue response. - Plant Sci. **119**: 79-96, 1996.

Martín, M., Urteaga, B., Sabater, B.: Chloroplast protein synthesis during barley leaf growth and senescence: effects of leaf excision. - J. exp. Bot. **37**: 230-237, 1987.

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

Mano, Y., Takahashi, H., Sato, K., Takeda, K.: Mapping genes for callus growth and shoot regeneration in barley (*Hordeum vulgare* L.). - Breed. Sci. **46**: 137-142, 1996.

Schenk, R.U., Hildebrandt, A.C.: Medium and techniques for induction and growth of monocotyledonous and dicotyledonous plant cell cultures. - Can. J. Bot. **50**: 199-204, 1972.

Vikrant, Rashid, A.: Somatic embryogenesis or shoot formation following high 2,4-D pulse-treatment of mature embryos of *Paspalum scrobiculatum*. - Biol. Plant. **46**: 297-300, 2003.

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

Zacchini, M., Rea, E., Tullio, M., Agazio, M.: Increased antioxidative capacity in maize calli during and after oxidative stress induced by a long lead treatment. - Plant Physiol. Biochem. **41**: 49-54, 2003.