African Journal of Biotechnology Vol. 10(84), pp. 19522-19533, 26 December, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB11.1736
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Development of long-term and reliable *in vitro* plant regeneration systems for elite malting barley varieties: Optimizing media formulation and explant selection

Gerardo Armando Aguado-Santacruz[1]*, Ángeles Velázquez-Ordinola[1], Blanca Moreno-Gómez[1], Luisa Marina Gómez-Torres[2], Luís Febronio Díaz-Espino[3] and Francisco Paúl Gámez Vázquez[4]

[1]Laboratory of Plant Molecular Physiology, Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias, Km 6.5 Carretera Celaya-San Miguel de Allende, Celaya, Guanajuato 38110, México.
[2]Departamento de Ingeniería Química y Ambiental. Universidad Nacional de Colombia, Avenida Carretera 30 No. 45-03, Bogotá, D.C., Colombia.
[3]Programa de Uso Eficiente de Agua, Campo Experimental Bajío, Instituto Nacional de Investigaciones Forestales y Agropecuarias, Km 6.5 Carretera Celaya-San Miguel de Allende, Celaya, Guanajuato 38110, México.
[4]Fundación Guanajuato Produce, Calle Nuevo León 603, Colonia Alameda, Celaya, Guanajuato 38050, México.

Accepted 7 December, 2011

The response to *in vitro* tissue culture of five important Mexican malting barley cultivars namely 'Armida', 'Esmeralda', 'Adabella', 'Esperanza' and 'Alina', was evaluated. Callus induction and plant regeneration were evaluated in shoot apices and immature barley embryos harvested eight, 12, 16, 20 and 24 days after pollination. These explants were cultured on 22 media formulations that included Murashige and Skoog (MS) and Chu (N6) media amended with two carbon sources and different concentrations of 2,4-Dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-2-methoxybenzoic acid (Dicamba), 6-benzyladenine (BA), casein hydrolyzate and L-proline. No regenerable callus lines could be recovered from the experiments using shoot apices as the starting material. Conversely, immature embryos harvested at 12 to 16 days after pollination (1.0 to 1.5 mm long) and cultured on formulations containing the basal MS medium supplemented with 2 mg L^{-1} of 2,4-D or Dicamba resulted in the production of nodular and friable embryogenic calli with regenerative capacity in all barley cultivars. A medium containing MS, Dicamba and maltose generated the highest percentage of embryogenic calli formation (2.1 in 'Adabella' vs. 23.4 in 'Alina' cultivar) with the maximal regenerative potential (7.2 in 'Armida' *vs.* 9.1 recovered plants per gram FW callus in 'Esperanza' cultivar). Using this medium, 'Esmeralda' embryogenic callus lines have been maintained for more than five years without loss of regenerability or the capacity for producing normal plants.

Key words: Barley, somatic embryogenesis, plant regeneration, immature embryos.

INTRODUCTION

Barley (*Hordeum vulgare* L.) is one of the most important cereal crops in the world along with maize (*Zea mays* L), wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.). Approximately 85% of the barley cultivated around the world is used as animal feed, and the rest is processed as malting and brewing grain or for human food (Kasha, 2007). Worldwide, barley is one of the most widely adapted grain cereals and is cultivated in all extreme climates, including subarctic areas and deserts (Baik and Ullrich, 2008). Currently, barley is gaining a renewed attention because of its high nutritional value, high

*Corresponding author. E-mail: gaguado@prodigy.net.mx or gaguados@gmail.com. Tel: +52-461-6115323 ext 122. Fax: +52-461-6115323 ext 141.

content of vitamins, trace minerals, dietary fiber and bioactive compounds, such as β-glucans, tocopherols, tocotrienols and phenolic compounds that are an excellent source of natural antioxidants for disease prevention (Baik and Ullrich, 2008; Gallegos-Infante et al., 2010).

With an annual production of 811,358 tons, barley is a crop of great social-economic importance in the central highlands of México (FAO, 2008). In México, barley is highly demanded by the malting industry because of the worldwide, well-recognized quality of Mexican beer. Considering that this crop is characterized by high genetic diversity, there is a great opportunity to identify cultivars and apply biotechnological tools for the genetic improvement of agronomic traits of barley for specific end purposes. 'Armida', 'Esmeralda', 'Adabella', 'Esperanza' and 'Alina' are elite malting barley cultivars developed by the National Institute for Forestry, Agriculture and Livestock Research of México. This elite barley germplasm was developed for the malting industry by traditional breeding and possesses outstanding agronomic traits, including high yield, grain of excellent industrial quality, adaptation to irrigation or rain-fed conditions and tolerance to some of the main diseases affecting this crop in México (Zamora-Díaz et al., 2008).

The development of efficient and reproducible *in vitro* tissue culture and plant regeneration protocols is a prerequisite for applying modern biotechnological tools for genetic improvement of crops, such as somaclonal variants recovery, production of transgenic and pathogen-free plants and clonal propagation of desirable germplasm, among others. However, barley is a recalcitrant species to *in vitro* tissue culture (Ganeshan et al., 2003). Consequently, there are only a few reports of commercial barley cultivars with consistent and efficient *in vitro* regenerable capacity (Cho et al., 1998; Ganeshan et al., 2006b; Dahleen and Manoharan, 2007), and these are principally North American and European cultivars. In addition, most of the economically important cultivars have low callus-induction efficiency (Abumhadi et al., 2005), and the *in vitro* plant regeneration capacity to yield green plants is limited to short periods (Mohanty and Ghosh, 1988). Part of this problem is related to the ample genetic diversity present in this crop, which results in a great variety of responses to *in vitro* tissue culture. Previous studies have shown that the establishment of barley embryogenic cultures and recovery of plants under *in vitro* conditions are controlled by several factors, including: a) plant genotype (Li et al., 2009; Tanasienko et al., 2009); b) type and physiological state of the explants (Ganeshan et al., 2003; Kasha, 2007); c) ethylene antagonists (Dahleen and Bregitzer, 2002; Chauhan and Kothari, 2004); d) plant-growth regulators (Ganeshan et al., 2006a; Gurel et al., 2009); e) carbohydrate sources (Walmsley et al., 1995; Wojnarowiez et al., 2004) and f) light conditions (Rikiishi et al., 2008).

In regard to the composition of culture media, growth regulators are fundamental to produce embryogenic calli with regenerative capacity. The auxins 2,4-Dichlorophenoxyacetic acid (2,4-D), 3,6-dichloro-2-methoxybenzoic acid (Dicamba) and 4-amino-3,5,6-trichloropicolinic acid (Picloram), alone or in combination with cytokinins such as 6-benzyladenine (BA), improve callus induction and maintenance in barley (Ziauddin and Kasha, 1990) and other cereals (Abumhadi et al., 2005). Cytokinins enhance plant differentiation and are commonly used in the regeneration medium for promoting shoot formation. Likewise, the use of optimal auxins/cytokinins ratios in the callus induction and sub-culturing media improves callus quality and regeneration capacity in cereals and grasses (Bhaskaran and Smith, 1990; Bai and Qu, 2001). Also, for the induction and establishment of regenerable callus cultures in barley, different explants containing meristematic cells such as leaf bases (Mohanty and Ghosh, 1988; Li et al., 2009), mature embryos (Gurel et al., 2009; Tanasienko et al., 2009), immature embryos (Halámková et al., 2004; Rikiishi et al., 2008) and shoot apical meristems (Ganeshan et al., 2003; Sticklen and Oraby, 2005), have been used. From these studies, it is now clear that in general, immature tissues respond faster than mature tissues, and that meristematic tissues tend to regenerate more efficient plants than the non-meristematic ones. In this connection, immature zygotic embryos of barley, as in other cereals (Vasil, 2005), have proved to be ideal explants for *in vitro* tissue culture, thereby allowing the establishment of callus, suspension and protoplasts cultures amenable to genetic manipulation (Dahleen and Bregitzer, 2002; Chang et al., 2003; Kasha, 2007).

In this study, we analyzed the morphogenic response of five elite malting barley cultivars to *in vitro* culture and determined the effect of explant type (immature embryo and shoot apices) and formulation composition. To the best of our knowledge, there are no protocols for *in vitro* plant regeneration of Mexican elite malting barley varieties, and none of the cultivars used in this study have been considered previously for analysis of *in vitro* plant regeneration capacity.

MATERIALS AND METHODS

Germplasm of five elite malting barley (*Hordeum vulgare* L.) cultivars namely 'Armida', 'Esmeralda', 'Adabella', 'Esperanza' and 'Alina', was utilized for *in vitro* tissue culture experiments. Shoot apices and immature embryos were utilized as the starting material to evaluate the morphogenic response of these five elite malting barley materials to *in vitro* culture.

Shoot apices

Seeds of the five barley cultivars were surface-sterilized by soaking them for 5 min in 70% (v/v) ethanol and for 20 min in 0.02% (w/v) aqueous mercuric chloride, followed by five rinses with sterile distilled water. Sterilized seeds were placed on MS medium

Table 1. Media utilized for induction of *in vitro* morphogenic responses from shoot apices of five malting barley cultivars.

Number	Media	Basal salt	2,4 D (mg L^{-1})	Picloram (1 mg L^{-1})	Adenine (mg L^{-1})	BA (mg L^{-1})	Casein hydrolyzate (500 mg L^{-1})
1	DA4B2-C	MS	1	—	40	2.0	Added
2	DA6B2-C	MS	1	—	60	2.0	Added
3	DA8B2-C	MS	1	—	80	2.0	Added
4	DA4B2	MS	1	—	40	2.0	—
5	DA6B2	MS	1	—	60	2.0	—
6	DA8B2	MS	1	—	80	2.0	—
7	025DB05	MS	0.25	—	—	0.50	—
8	2DB025	MS	2.0	—	—	0.25	—
9	PA40B2-C	MS	—	Added	40	2.0	Added
10	PA40B2	MS	—	Added	40	2.0	—
11	3DB1	MS	3.0	—	—	1.0	—
12	4DB1	MS	4.0	—	—	1.0	—
13	3D	MS	3.0	—	—	—	—
14	4D	MS	4.0	—	—	—	—

Table 2. Media utilized for induction of *in vitro* morphogenic responses from immature embryos of five malting barley cultivars.

Number	Media	Basal salt	Carbohydrate (30 g L^{-1})	Auxin (2 mg L^{-1})	L-proline (2.3 g L^{-1})
1	MMD-P	MS	Maltose	2,4 D	Added
2	MMD	MS	Maltose	2,4 D	—
3	MMDi-P	MS	Maltose	Dicamba	Added
4	MMDi	MS	Maltose	Dicamba	—
5	NSD-P	N6	Sucrose	2,4 D	Added
6	NSD	N6	Sucrose	2,4 D	—
7	NSDi-P	N6	Sucrose	Dicamba	Added
8	NSDi	N6	Sucrose	Dicamba	—

(Murashige and Skoog, 1962) that was solidified with 2.5 g L^{-1} of gelrite. Shoot apices were isolated from three to five day-old seedlings and used as the starting material. To isolate these explants, seeds were germinated in the dark to promote elongation of the subcoleoptilar internode, and then 3-cm long segments containing the coleoptilar node were excised from seedlings and placed on Petri dishes containing the different media formulations.

Immature embryos

Inflorescences were collected during the winter season from field-grown barley plants. Immature embryos from the different cultivars were harvested eight, 12, 16, 20 and 24 days after pollination. The immature seeds were surface-sterilized by soaking them for 5 min in 70% (v/v) ethanol and for 20 min in 0.02% (w/v) aqueous mercuric chloride and finally rinsing them five times with sterile distilled water. Immature embryos (0.3 to 1.5 mm long) were excised from seeds and cultured on the different media formulations with the embryonary axis facing down. Culturing the immature embryos in this way has been instrumental for successful *in vitro* morphogenic response of cereals such as maize (Aguado-Santacruz et al., 2007).

Media tested for induction of morphogenic responses in barley

Shoot apices and immature embryos were placed on Petri dishes containing 30 ml of the different induction media. 15 Petri dishes containing eight shoot tips or immature embryos (replications) for every barley cultivar/medium studied were evaluated. Shoot apices were cultured on 14 media formulations containing the MS basal vitamins and salts, 0 to 4 mg L^{-1} 2,4-D, 0 or 1 mg L^{-1} Picloram, 0 to 80 mg L^{-1} adenine and 0 to 2 mg L^{-1} BA, 0 or 500 mg L^{-1} casein hydrolyzate and 2.5 g L^{-1} gelrite (Table 1). For immature embryos, tested media components included the basal salts and vitamins of the N6 (Chu et al., 1975) or MS media (Murashige and Skoog, 1962), 0 or 2.3 g L^{-1} L-proline, 30 g L^{-1} sucrose or maltose, 2 mg L^{-1} 2,4-D or Dicamba and 8 g L^{-1} bacto agar (Table 2). All media were adjusted to a pH of 5.8 and then sterilized at 121°C and 15 psi for 15 min. Frequencies of callus induction were quantified 45 days

after plating the explants on the different induction media.

Callus maintenance and plant regeneration

Individual callus lines were established by dissecting and sub-culturing the initially formed callus masses on the same induction media for three sub-cultures (30 days for each one) in darkness or semidarkness (photon flux = 7 µmol m^{-2} s^{-1}) at 25 ± 1°C and 13% relative humidity. To determine the capacity of the individual cell lines to regenerate into whole plants, part of the multiplied calli was transferred to Petri dishes containing full- or half-strength MS and then placed in a growth room at 25 ± 1°C, at a 13% relative humidity and an 8-h photoperiod (photon flux = 40 µmol m^{-2} s^{-1}). Subsequently, 1- to 2-cm height-shoots were transferred to 400-ml GA7 Magenta boxes containing 50 ml of half-strength MS amended with 0.5 or 1.0 mg L^{-1} indole acetic acid (IAA) and maintained under the previously mentioned environmental conditions until they reached heights of 7 to 9 cm and developed three to five roots with a length of 2 to 4 cm.

MS and N6 basal salts, sucrose and maltose were purchased from Phyto Technology (Shawnee Mission, KS). Growth regulators, L-proline, and gelrite were acquired from Sigma-Aldrich (St. Louis, MO). Bacto agar was acquired from BD Biosciences (Franklin Lakes, NJ).

Acclimatization and hardening of the regenerated plants

When *in vitro* regenerated-plantlets reached the projected developmental stage, they were removed from the Magenta boxes, and their roots were thoroughly rinsed with running water to eliminate residual media. Subsequently, the plantlets were transferred into half-liter pots containing a mixture of sterile clayey soil and sand (50:50), covered with transparent polyethylene bags and placed in a growth chamber at 21 ± 1°C, at a 13% relative humidity and at a photon flux of 70 µmol m^{-2} s^{-1}. After 15 to 20 days of acclimation in the growth room and when they were 15 cm in height, plants were transferred to the greenhouse and fertilized with 10 g of a NPK chemical fertilizer mixture (17:17:17). Finally, when plants were 20 to 25 cm in height, they were transferred to the field where two more NPK fertilizing doses were applied.

Statistical analysis

Significant differences between the frequencies of calli induction among different embryo ages, as well as in the frequencies of embryogenic callus formation and plant regeneration among the different media, were evaluated through one-way analysis of variance. Means were separated using Tukey's tests (Zar, 1999).

RESULTS

In vitro response of shoot apices

One week after their culture on the different media in the darkness at 25°C, the shoot apices continued their development and new leaves appeared. Hence, the shoot apices had to be re-dissected to eliminate these new forming leaves and were then placed back on the same induction media. The apices were sub-cultured monthly during two consecutive periods. Independent of the media formulation tested, all shoot apices became necrotized at the second sub-culture, while at 60 days after callus initiation, the explants started to die.

In vitro response of immature embryos

As opposed to shoot apices, when immature embryos were utilized as the starting material, all barley cultivars formed callus with efficiencies varying as a function of plant genotype, media formulation and explant age (Figures 1 and 2). All immature embryos cultured on MS or N6 media supplemented with or without proline and containing one of two different carbohydrate and auxin sources, formed callus rapidly (10 days) after plating on the induction media (Figure 3a). It was evident that medium NSD containing N6 salts, 2,4-D and sucrose, resulted in a lower frequency of callus induction in four of the five barley cultivars studied, namely 'Armida', 'Esmeralda', 'Adabella' and 'Alina'; the addition of proline to the NSD-P culture medium did not improve the immature embryo response (Figures 1 and 2). A highly contrasting response, however, was observed in the 'Esperanza' cultivar, with no callus formation in 20-days embryos and a severely reduced percentage of callus formation for embryos collected 24 days after pollination (Figure 2).

Overall, the culture of eight-day immature embryos resulted in the lowest frequency of callus induction in all cultivars, with 'Esmeralda' germplasm cultured on MMD medium containing MS, maltose and 2 mg L^{-1} 2,4-D developing the highest percentage of callus formation at this embryo age (48%; Figure 1). Four more days in the developmental stage of the embryos increased the percentage of callus formation considerably; values obtained in 12-day embryos ranged from 21% for 'Armida' embryos cultured on NSD-P medium to 100 and 99% for 'Adabella' and 'Alina' embryos cultured on MMD and MMDi media, respectively (Figures 1 and 2). Maximum callus induction frequencies in the 'Esperanza' cultivar were clearly obtained in immature embryos collected at 16 and 20 days after pollination, independent of the media formulation employed. In the rest of the cultivars, these frequencies varied as a function of the medium tested, although embryos harvested between 12 and 24 days after pollination seemed to be, in general, amenable for calli induction.

Regenerative competence of the induced callus

After achieving an efficient calli induction frequency by managing plant genotype, immature embryo age and media formulation, the competence for plant regeneration of all multiplied germinal lines was further tested. During the induction experiments, different callus morphologies were obtained. Younger and smaller immature embryos (1.0 to 1.5 mm) formed regenerable embryogenic calli

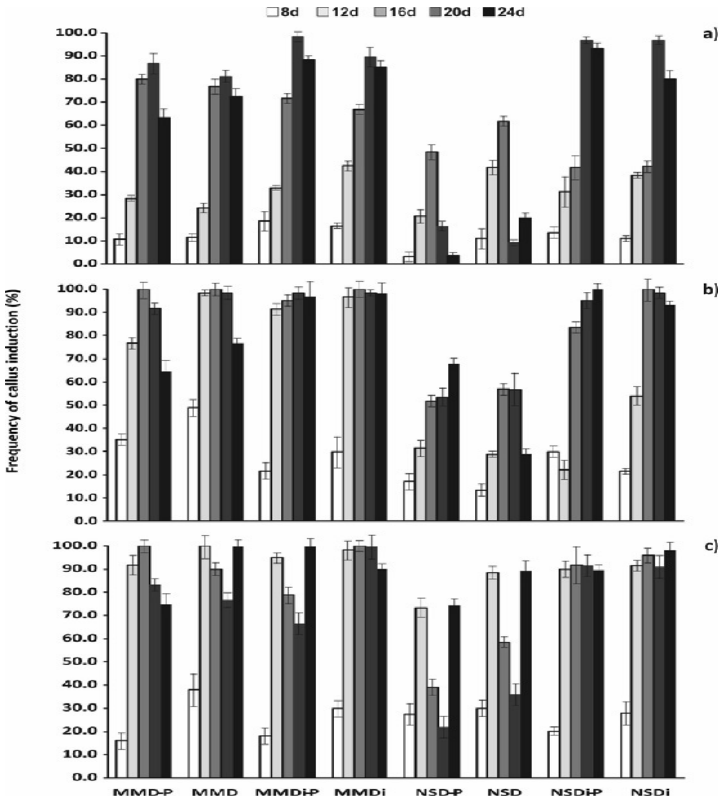

Figure 1. Effect of immature embryo age (days after pollination) and medium composition on frequency of callus induction (%) in five elite malting barley cultivars. a) Armida; b) Esmeralda; c) Adabella.

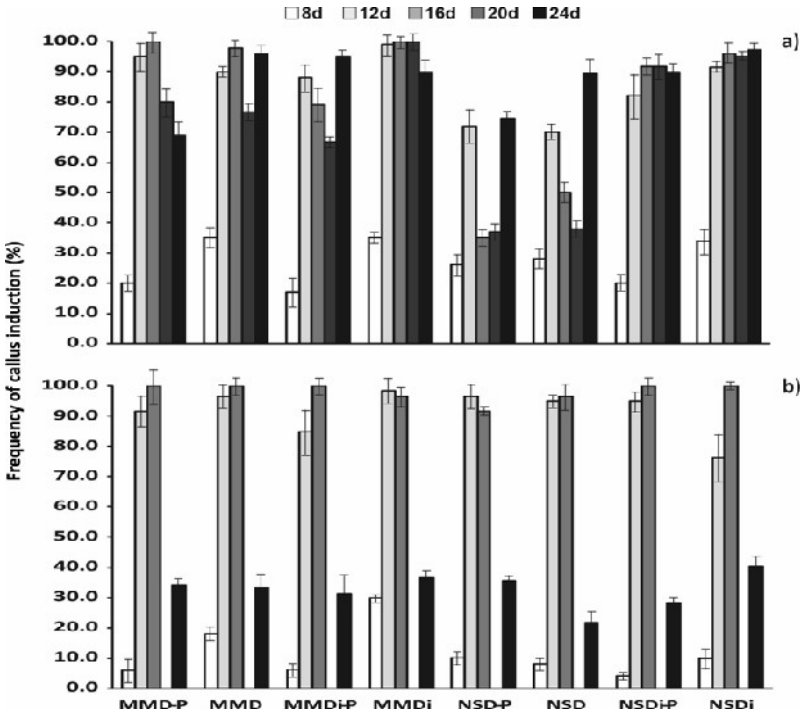

Figure 2. Effect of immature embryo age (days after pollination) and medium composition on frequency of callus induction (%) in five elite malting barley cultivars. a) Alina; b) Esperanza.