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

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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

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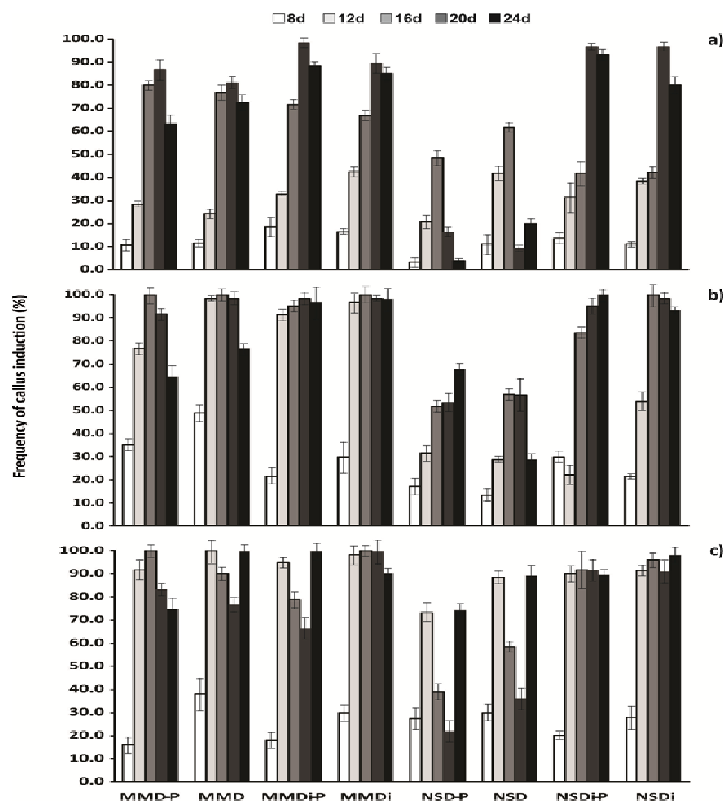
Figure 1. Effect of immature embryo age (days after pollination) and medium composition on frequency of callus induction (%) in five elite malting barley cultivars. a) Armida; b) Esmeralda; c) Adabella.
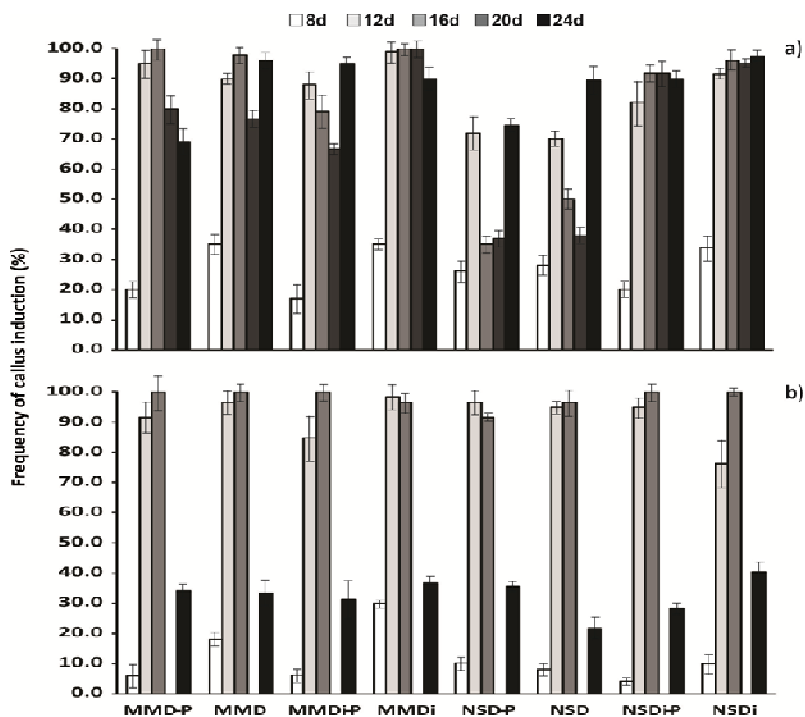

Figure 2. Effect of immature embryo age (days after pollination) and medium composition on frequency of callus induction (%) in five elite malting barley cultivars. a) Alina; b) Esperanza.
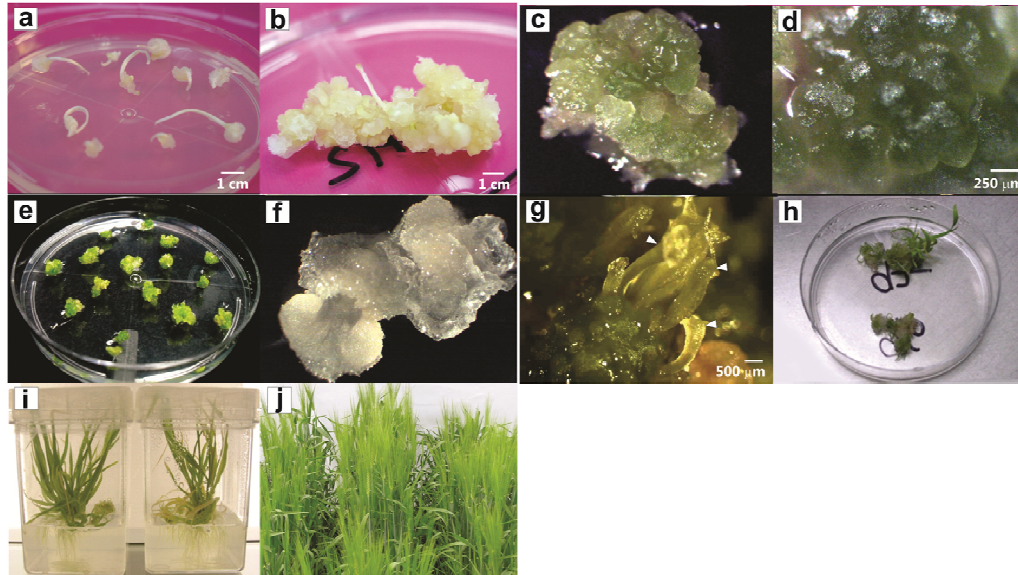
Figure 3. Embryogenic callus induction and plant regeneration in elite malting barley varieties. a) 16-day immature embryos of 'Esmeralda' cultivar after 10 days of culture on medium MMDi; b) Friable, highly embryogenic callus derived from immature embryos cultured on medium MMDi-P containing MS basal salts, Dicamba and maltose; c) 'Esmeralda' embryogenic callus cultures after 10 days of transference to the light; d) Close-up of green embryogenic calli showing a nodular structure; e) Propagation of the fast-growing, green and regenerable embryogenic callus cultures; f) Non-morphogenic callus obtained from the *in vitro* culture of immature embryos; g) Close-up of green, germinated somatic embryos showing a trumpet-like appearance (arrows); h) Shoot formation in embryogenic calli cultured in the light on hormone-free medium; i) Strong rooting of shoots transferred to GA 7 Magenta boxes containing full strength MS amended with 0.5 mg L^{-1} IAA; j) Mature regenerated plants of elite malting barley varieties established on the field.

whose structure ranged from hard, compact, nodular and yellowish to soft, watery, loose, friable and creamy white to translucent (Figure 3b). When transferred to the light, the nodular embryogenic calli turned green (Figures 3c to e), exhibited a better growth and did not initiate the regenerative process if sub-cultured every 25 to 30 days on the same induction media. Although most of the older (>20 days after fecundation) and larger (>1.5 mm) immature embryos were able to produce callus, a great part of this material was not stable, generated roots and showed no whole-plant regeneration competence; appearance of the non-morphogenic calli was predominantly soft and spongy (Figure 3f). After being transferred to media containing a full- or half-strength MS medium and then cultured on light conditions, regenerable embryogenic calli turned green, germinated and formed trumpet-like embryos (Figure 3g). Subsequently, these somatic embryos developed shoots (Figure 3h) and then formed roots. No albino plantlets have been recovered from the different evaluated media.

Although shoots spontaneously formed roots after their transference to full- or half-strength MS medium, the incorporation of IAA into the media fostered root development (Figure 3i). After analysis of the regenerative capacity of the induced callus was carried out, it was evident that a very low percentage of the induced calli had regenerative competence (Table 3). 'Armida' was the cultivar that showed the best response over all media tested in terms of regenerable calli produced. At relatively low or high efficiencies, MMD and MMDi were the only media that functioned over all barley varieties tested; proline addition seemed to reduce the efficiency of the formulations to generate regenerable calli. In general terms, media based on an N6 basal medium were less efficient in producing regenerative calli than media based on MS basal medium. In particular, no regenerative calli could be recovered from the NSDi medium that was amended with sucrose and Dicamba. As observed with the MS background medium, addition of L-proline to the NSDi-P medium did not improve the morphogenic response of the induced calli. Furthermore, as a final step within the evaluation of the capacity of the media formulations tested for inducing morphogenic responses in barley cultivars, the regeneration efficiency of the induced calli was measured in terms of plantlets regenerated per gram of fresh weight callus (Table 4). This analysis show that the efficiency of recovered plantlets per gram of fresh material ranged from 0 on media NSDi-P and NSDi to 9.1 on the MMDi medium; maximal plantlet recovering percentages were obtained on this latter medium, which contained MS, maltose and Dicamba (Table 4).

Table 3. Efficiency of regenerable callus formation (%) from immature zygotic embryos of five malting barley cultivars.

Media	Cultivar				
	'Armida'	'Esmeralda'	'Adabella'	'Alina'	'Esperanza'
MMD-P	2.2[d 1_/]	0.0[d]	0.0[e]	0.0[d]	0.0[b]
MMD	4.3[c]	4.0[b]	14.7[a]	13.3[b]	2.1[a]
MMDi-P	2.2[d]	1.8[c]	0.0[e]	0.0[d]	0.0[b]
MMDi	16.8[a]	19.2[a]	2.1[d]	23.4[a]	2.2[a]
NSD-P	9.7[b]	0.0[d]	7.1[b]	0.0[d]	0.0[b]
NSD	2.0[d]	0.0[d]	4.3[c]	8.2[c]	0.0[b]
NSDi-P	0.0[e]	0.0[d]	0.0[e]	0.0[d]	0.0[b]
NSDi	0.0[e]	0.0[d]	0.0[e]	0.0[d]	0.0[b]

[1_/] Means followed by different letters are significantly different (P≤0.05) among media formulations. Regenerable callus formation efficiency was calculated as the no. of regenerable calli/no. of induced calli x 100. Experiments were performed at least twice.

Table 4. Regeneration efficiency (plantlets per g FW callus) from calli induced on 15 different media formulations.

Media	Cultivar				
	'Armida'	'Esmeralda'	'Adabella'	'Alina'	'Esperanza'
MMD-P	3.7[cd 1_/]	0.0[d]	0.0[c]	0.0[c]	0.0[c]
MMD	3.1[d]	2.4[c]	2.8[b]	3.8[b]	4.2[b]
MMDi-P	4.9[b]	5.9[b]	0.0[c]	0.0[c]	0.0[c]
MMDi	7.2[a]	7.3[a]	7.9[a]	8.4[a]	9.1[a]
NSD-P	4.5[bc]	0.0[d]	3.7[b]	0.0[c]	0.0[c]
NSD	4.2[bc]	0.0[d]	3.8[b]	3.5[b]	0.0[c]
NSDi-P	0.0[e]	0.0[d]	0.0[c]	0.0[c]	0.0[c]
NSDi	0.0[e]	0.0[d]	0.0[c]	0.0[c]	0.0[c]

[1_/] Means followed by different letters are significantly different (P≤0.05) among media formulations. Experiments were performed at least twice.

Acclimatization and hardening of the regenerated plants

After the 15-day acclimation period of the plantlets in the growth chamber, plants were transferred to the greenhouse where they terminated to consolidate their hardening process. Subsequently, barley plants 20 to 25 cm in height were removed from the pots and then planted in the field and chemically fertilized twice. All plantlets transferred to the field established successfully, developed to maturity and set seeds (Figure 3j).

DISCUSSION

The well-known recalcitrance of cereals and grasses to *in vitro* culture has been overcome by recognizing the central role of plant genotype and by manipulating primary factors affecting response to tissue culture, such as media constituents, type and development stage of explant and general environmental growing conditions. Considering all these factors, great advances have been achieved within the last decade in the research of *in vitro* culture and genetic manipulation of barley and cereals in general (Aguado-Santacruz et al., 2007). Although several protocols for *in vitro* culture of barley have been developed (Ruíz et al., 1992; Vitanova et al., 1995; Walmsley et al., 1995; Barro et al., 1999; Li and Devaux, 2001; Bregitzer et al., 2002; Dahleen and Bregitzer, 2002; Chang et al., 2003; Ganeshan et al., 2003; Chauhan and Kothari, 2004; Chernobrovkina et al., 2004; Halámková et al., 2004; Klcova et al., 2004; Sahrawat and Chand, 2004; Serhantova et al., 2004; Sharma et al., 2004; Tidema and Truve, 2004; Sharma et al., 2005; Ganeshan et al., 2006b; Dahleen and Manoharan, 2007; Jha et al., 2007; Sharma et al., 2007; Li et al., 2009; Tanasienko et al., 2009), only a small number of barley genotypes have been identified as possessing regenerative capacity (Lemaux et al., 1999), and many of them have low economic importance. Genetic differences in the potential barley cultivars used for whole-plant regeneration (Kasha, 2007) emphasize the importance of analyzing the response of particular elite barley materials to *in vitro* culture in order to define the specific growth conditions necessary to generate the totipotent material required for applying the potential tools of the *in vitro*

technology, such as genetic transformation, somaclonal variants recovery, somatic hybridization, and molecular farming, among others.

Finding suitable barley responses to *in vitro* culture involves the correct selection of the explants (and their proper developmental stage) to be used as the starting material. Immature embryos (Walmsley et al., 1995; Przetakiewicz et al., 2003) and shoot apices (Sticklen and Oraby, 2005) have been shown to be suitable explants to induce *in vitro* cultures from barley and other cereal crops. In this work, it was not possible to obtain callus cultures from shoot apices of barley. Even though these explants have been suitable to obtain regenerative material from other barley cultivars explants (Zhang et al., 1999; Ganeshan et al., 2003; Przetakiewicz et al., 2003), in this work shoot apices from all tested cultivars acquired a brownish and necrotic appearance soon after they were plated. Thus, shoot apices seem not to be suitable explants for initiating tissue cultures from the elite malting cultivars evaluated here. Alternatively, when MS is utilized as the background medium, the concentrations and the balance of the growth regulators utilized in this study might not be suitable for induction of suitable morphogenic responses from shoot apices in these genotypes. Our results are in contrast with those reported by Ganeshan et al. (2003), who successfully induced callus from leaf-base/apical meristems cultures on media containing 1 mg L^{-1} of 2,4-D, casein hydrolyzate and L-proline, but with the addition of high levels of $CuSO_4$, a salt that seems to improve the morphogenic response of barley to *in vitro* culture (Tanasienko et al., 2009). Likewise, Vitanova et al. (1995) reported higher embryogenic callus induction and regeneration rates when seedlings (leaf bases with the complete apical meristem) were grown in the presence of high concentrations of 2,4-D (8 mg L^{-1}).

Additional research is necessary to identify critical components and conditions for the induction of morphogenic regenerable responses in these elite malting varieties using shoot apices in light of the advantages of using this type of explant as the starting material. For example, the possibility of using this type of explant throughout the year, easy isolation in a short period (within three to five days of germination of mature seeds) and the elimination of the necessity of growing the donor plants to obtain immature materials (Ganeshan et al., 2003), which, in turn, reduces the time required to regenerate complete plants, makes this explant an attractive option for initiation of callus cultures. Additionally, shoot apices-derived calli permit the clonal propagation of useful germplasm.

Effect of immature embryo size and age on regenerable callus induction and plant regeneration competence

The size and age of immature embryos were important factors for obtaining suitable morphogenic responses in the elite malting barley cultivars analyzed in this study. Immature embryos were able to form callus even eight days after pollination. This coincides with the fact of callus forming from the embryo scutellum (Golds et al., 1993), whose development starts at approximately eight days after pollination and finishes approximately 21 days after fecundation in barley (Rikiishi et al., 2003). Next, callus induction in the elite malting cultivars was conserved at a relatively high level until 20 days and diminished slightly at 24 days after pollination. Once formed, differences in the regeneration competence of the induced calli were evident. Maximal competence for regenerable callus formation was observed in 1- to 1.5-mm long embryos harvested from 12 to 16 days after pollination (data not shown). These results are consistent with other reports where high rates of embryogenic callus formation were obtained from immature embryos collected 14 days after pollination (Ruíz et al., 1992; Rikiishi et al., 2003). Although immature embryos in the range of 1 to 3 mm are the most common explants for barley regeneration and transformation research (Wan and Lemaux, 1994; Bregitzer et al., 1998), other studies have found that smaller (0.5 to 1.5 mm) embryos generate calli with the highest regeneration potential (Chang et al., 2003).

The morphology of the regenerable calli generated in this study from the different malting barley varieties indicated that they were fundamentally embryogenic in nature. The structure of regenerable embryogenic calli derived from younger and smaller immature embryos (1.0 to 1.5 mm) ranged from hard, compact, nodular and yellowish to soft, watery, loose, friable and creamy white to translucent (Figure 3b). This morphology was in essence, similar to that described by other authors. For example, Bregitzer (1992) described embryogenic calli as morphogenic structures ranging from white, finely dispersed and relatively friable to more compact, nodular, yellowish-white, and non-friable masses. Ziauddin and Kasha (1990) and Kott et al. (1987) referred to embryogenic calli as opaque and nodular masses with a high potential for production of somatic embryos. According to Bregitzer (1992), healthy, vigorously growing callus cultures tend to be more friable within particular genotypes. More also, Castillo et al. (1998) and Senarath (2007) described the same embryogenic calli morphology and concluded that successful embryogenic callus initiation is highly dependent on the cultivar and the auxin used. Vitanova et al. (1995) and Ganeshan et al. (2006b) found embryogenic calli as very hard, compact and white nodular masses with a high regeneration frequency, while Nonohay et al. (1999) described embryogenic calli as compact and smooth, showing small and dense cells with minuscule vacuoles, anatomical characteristics common to all embryogenic systems. Conversely to the regenerable embryogenic masses, the appearance of the non-regenerable calli found in our

study was predominantly soft and spongy (Figure 3f).

Effects of basal salts, balance of hormones and carbohydrate sources on regenerable callus induction and plant regeneration competence

Culture medium was critical for successful callus induction and plant regeneration in all tested malting barley varieties. Our results demonstrate that MS functioned better than N6 as a background medium for induction of regenerable calli. This observation agreed with earlier works in which the MS medium was utilized successfully for *in vitro* regeneration of barley (Bregitzer, 1992; Chang et al., 2003; Przetakiewicz et al., 2003; Chernobrovkina et al., 2004). In contrast, the N6 medium was not favorable for the induction of morphogenic responses in barley, even when this medium has proven to be suitable for anther culture of cereals (Chu, 1978), establishment of efficient regenerable tissue cultures of maize from immature embryos (Bohorova et al., 1995; Zacchini et al., 2003; Aguado-Santacruz et al., 2007) and plant regeneration from barley microspores (Ritala et al., 2001). Differences observed in the morphogenic efficiencies between MS and N6 media might be attributed to their respective inorganic nitrogen components and the favorable NH^{3+}/NO_3^- ratio present in the MS basal formulation. The MS medium contains 60 mM of inorganic nitrogen, compared to 35 mM in N6 (Armstrong and Green, 1985), while the ammonium: nitrate ratio is approximately 1:2 in MS compared with 1:4 in N6 (Samoylov et al., 1998). These differences in the balance of the inorganic salts seem to be important for achieving efficient somatic embryogenesis and plant regeneration in mexican elite barley cultivars.

The addition of organic nitrogen in the form of different amino acids has been shown to enhance differentiation and somatic embryogenesis in cereals because early stages of embryogenesis require the presence of these molecules, which can be readily incorporated into proteins. Later on, at the maturation and regeneration stages, requirements of organic nitrogen decrease (Nuutila et al., 2000). However, in the case of barley, previous studies have shown that the addition of proline at 160 mg L^{-1} has a negative impact on the *in vitro* morphogenic response of this cereal (Chernobrovkina et al., 2004). Accordingly, in our study, the addition of this amino acid at even higher concentrations (2.3 g L^{-1}) did not improve the morphogenic response of the evaluated malting barley varieties.

Furthermore, concentration and balance of plant-growth regulators, auxins and cytokinins, play the most important role in embryogenic callus induction, and their effect on plant regeneration in barley cultures have been widely investigated (Hanzel et al., 1985; Walmsley et al., 1995; Barro et al., 1999; Przetakiewicz et al., 2003; Serhantova et al., 2004). In general, media for the

induction and proliferation of callus in *Poaceae,* and particularly barley, require relatively high concentrations of auxins; commonly 2,4-D, Dicamba and Picloram are the preferred ones. 2,4-D concentrations in the range of 1 to 3 mg L^{-1} are efficient for the induction of embryogenic regenerable calli, while Dicamba and Picloram work better in the range of 1.5 to 4 mg L^{-1} (Halámková et al., 2004), and 2 to 6 mg L^{-1} (Barro et al., 1999), respectively. In our case, the use of both 2,4-D and Dicamba resulted in embryogenic friable calli possessing whole-plant regeneration competence after transfer to a medium lacking growth regulators. The results of our investigation show that a concentration of 2 mg L^{-1} of 2,4-D or Dicamba was adequate for callus induction. These results agree with findings from other authors. For example, Walmsley et al. (1995) stated that 2 mg L^{-1} of 2,4-D was suitable for initiation of embryogenic callus in three barley varieties, while Chang et al. (2003) found no advantages towards callus induction and plant regeneration by using concentrations of 2,4-D or Dicamba higher than 3 mg L^{-1}. Optimizing hormone concentrations is important because the use of excessive concentrations can result in somatic mutations (Choi et al., 2001). Thus, determining the minimal concentration of hormones, as well as the proper balance of the auxin/cytokinin ratio required for induction of embryogenic regenerable cultures is essential for the preservation of the identity of the original material, especially for long-term calli maintenance purposes.

Our results demonstrate that the embryogenic potential of the barley germplam was best expressed when Dicamba was used over 2,4-D. This observation is in agreement with results obtained from other authors working with barley. In this work, it was found that Dicamba was a more effective elicitor of callus induction, somatic embryogenic responses, maintenance of embryogenic callus and regeneration potential (Castillo et al., 1998; Przetakiewicz et al., 2003; Halámková et al., 2004; Tidema and Truve, 2004; Gurel et al., 2009). Likewise, Trifonova et al. (2001) reported that compared to 2,4-D, Dicamba is a better hormonal agent for the regeneration of transgenic barley. The use of 2,4-D in the long-term maintenance of barley cultures causes a loss of the regenerative potential of the embryogenic masses over time (Bregitzer et al., 1995).

Carbohydrate source is also another important factor that influences somatic embryogenesis in plant tissue culture. According to our results, maltose apparently interacted positively with MS and Dicamba, favoring the embryogenic response of barley. Experiments in which maltose was replaced by sucrose in media containing MS and Dicamba showed a decrease in the percentage of regenerable calli obtained from the culture of immature embryos of barley (data not shown). This finding was consistent with previous evidence provided by other authors demonstrating the advantage of using maltose as the carbon source in different formulations (Sharma et al.,

2004; Abumhadi et al., 2005; Ganeshan et al., 2006b; Sharma et al., 2007). The beneficial effect of maltose on embryogenic callus induction in other cereals has been attributed to its influence on cell differentiation, cell metabolism and viability (Brisibe et al., 1994; Indrianto et al., 1999). Compared to other carbohydrates, this sugar is metabolized more slowly than sucrose and may provide a readily metabolizable energy source over a longer period of culture. Furthermore, some authors relate its positive effect on the somatic embryogenesis of barley to a starvation stress caused by a slowing hydrolysis of maltose to glucose and to maintenance of a constant osmolality of the culture medium over time (Zhou et al., 1991; Indrianto et al., 1999).

Further refinements of the media formulations proposed here will require testing of other components previously reported as having a positive impact on somatic embryogenesis and shoot regeneration in barley, such as adding ions of Ag^+, Co^{2+}, Ni^{2+}, Zn^{2+} and Cu^{2+} to the culture medium. These ions function as ethylene antagonists (Castillo et al., 1998; Cho et al., 1998; Dahleen and Bregitzer, 2002) and have a dramatic effect on the improvement of somatic embryogenesis and shoot regeneration competence in barley tissue cultures.

Long-term regenerable cultures

Some of the embryogenic callus lines generated here from the 'Esmeralda' cultivar using the MMDi medium have been maintained for more than five years without losing their embryogenic nature, regenerative capacity or potential for producing fertile and normal barley plants. The main problem in barley tissue culture is maintaining the regeneration potential of the cultures for at least three to five months, while preventing the formation of abnormal plants (Kasha, 2007). Consequently, there are very few published reports in which the embryogenic and regenerative potential of barley cultures are retained for more than seven months (Weigel and Hughes, 1985; Ziauddin and Kasha, 1990; Bregitzer and Poulson, 1995; Castillo et al., 1998; Cho et al., 1998; Dahleen and Bregitzer, 2002; Chang et al., 2003). Although Weigel and Hughes (1985) developed long-term barley callus cultures, which retained their regenerative capacity for more than three years after initiation, to the best of our knowledge the barley callus lines generated here are unique in that they have maintained their regenerative potential for more than five years.

Different approaches have been proposed to circumvent the detrimental effects of continuous culturing of callus lines. Bregitzer et al. (1998) mentioned that compared to 2,4-D, Dicamba seems to increase the probability of mutations in barley tissue culture, while Vitanova et al. (1995) concluded that the morphogenic response of the primary calli could be maintained until the third sub-culture, despite using high concentrations of 2,4-D in the medium. To reduce the probability of somatic mutations, Choi et al. (2001) recommended to sub-culture the barley callus lines only six times, especially when high concentrations of 2,4-D are used, whereas Chang et al. (2003) mentioned that four to five sub-cultures of callus grown on modified MS medium with 3 mg L^{-1} of 2,4-D or Dicamba would provide enough and suitable target material for genetic transformation studies without a high risk of mutations. Furthermore, the addition of 0.1 mg L^{-1} BA to the sub-culture medium is sometimes essential for maintenance of barley embryogenic lines for more than a year without a marked loss in the regenerability or competence for normal plant formation (Cho et al., 1998).

Therefore, we expect that the use of MMDi medium with suitable elite malting barley genotypes can fulfill some of the requirements for the successful application of the different biotechnological tools, such as production of pathogen-free plants and propagation of desirable germplasm or genetic transformation, in which the generation of a fast-growing and highly embryogenic material capable of retaining its regenerative capacity and potential for production of normal and fertile plants for long periods of time is an essential prerequisite.

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Abbreviations:

2,4-D, 2,4-Dichlorophenoxyacetic acid; **Dicamba,** 3,6-dichloro-2-methoxybenzoic acid; **Picloram,** 4-amino-3,5,6-trichloropicolinic acid; **BA,** 6-benzyladenine, **MS,** Murashige and Skoog medium; **N6,** Chu medium; **IAA,** indole acetic acid

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