EMBRYOGENIC CALLUS INDUCTION ON THE SCUTELLUM AND REGENERATION OF PLANTS AS BASIS FOR GENETIC TRANSFORMATION OF SPRING WHEAT (*TRITICUM AESTIVUM* L.) CULTIVARS FROM ARGENTINA

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ABSTRACT

An effective tissue culture is an indispensable basis for the production of transgenic plants. In this study, we investigated the induction of embryogenic callus production from the scutellum of spring wheat and subsequent regeneration of plants. Twenty-two Argentine spring wheat varieties from three breeders were screened to select promising genotypes for the optimization of the procedure. Experimental variables were the developmental stage of the immature embryos and the concentration of the growth regulator 2,4-dichlorophenoxyacetic acid (2,4-D). Two developmental stages, termed H and W, respectively (according to He *et al.*, 1986), were selected, and their performance in culture media containing high and low concentrations of 2,4-D was examined. Parameters for the screening were: callus induction, callus proliferation on the scutellum, precocious germination of the immature embryo (as negative trait), regeneration and shoot formation and total efficiency of the *in vitro* culture. The best results were achieved with a combination of developmental stage H and a low concentration of 2,4-D. Scutellar callus induction and callus proliferation on the scutellum surface were positively correlated. However, scutellar callus induction, regeneration and number of shoots were independent of each other. Considerable genotype differences were found in the suitability of the varieties investigated for spring wheat propagation via *in vitro* culture. The cultivar *Klein Brujo* exhibited the highest overall culture efficiency (84.4 %) related to the number of immature embryos used and the number of plants regenerated from one embryo (9.5).

Key words: *In vitro* culture, immature embryos, embryogenic scutellar callus, callus proliferation

RESUMEN

La base indispensable para la producción de plantas transgénicas es un eficaz cultivo de tejidos. En este estudio se investigó la inducción de callo embriogénico y su posterior regeneración en trigo. Se analizaron veintidós variedades argentinas de trigo de primavera de tres criadores, para seleccionar los genotipos adecuados para la optimización del procedimiento. Las variables experimentales fueron la etapa de desarrollo de embriones inmaduros y la concentración de ácido 2,4-diclorofenoxiacético (2,4-D), un regulador del crecimiento. Se seleccionaron dos etapas del desarrollo denominadas H y W, respectivamente (de acuerdo a He *et al.*, 1986) y se examinaron sus comportamientos en los medios de cultivo con concentraciones altas y bajas de 2,4-D. Los parámetros del análisis fueron: inducción de callo, proliferación de callo escutelar, germinación precoz del embrión (rasgo negativo), regeneración y formación de plántulas y eficiencia del cultivo *in vitro*. Los mejores resultados se obtuvieron con una combinación de la etapa de desarrollo H y una concentración baja de 2,4-D. Hubo una correlación positiva entre la inducción de callo y su proliferación escutelar. Sin embargo, la inducción de callo, la regeneración y el número de plántulas fueron independientes entre sí. Las variedades de trigo investigadas mostraron considerables diferencias genotípicas en su capacidad de propagación en cultivo *in vitro*. La variedad *Klein Brujo* exhibió la eficiencia más alta de cultivo (84,4%), en relación con el número de embriones utilizados y de plantas regeneradas (9,5) a partir de un embrión.

Palabras clave: Cultivo *in vitro*, embriones inmaduros, callo embriogénico escutelar, proliferación de callo

INTRODUCTION

Wheat is one of the world's major food crops. Unfortunately, stable transformation efficiency is rather erratic and not very high. For successful biotechnological work with wheat, methodological improvements, such as an efficient system with a high potential of regeneration, are required. Although *in vitro* techniques have been partly established for wheat, many of the factors that affect the development of embryogenic callus and subsequently regulate plant regeneration are not yet well understood. As compared to model genotypes like Florida and Bobwhite (Rasco-Gaunt *et al.,* 2001), high-yield cultivars of wheat perform poorly in both processes (León *et al.,* 2006). As starting material for tissue culture, wheat offers only a few sources of explants that are suitable for regeneration in tissue culture (Sparks and Jones, 2009), e.g. immature zygotic embryos and immature inflorescences. The scutellum surface of immature zygotic embryos is the target most commonly used for wheat genetic transformation with particle bombardment or gene transfer by *Agrobacterium* (Sparks and Jones, 2009). Calli proliferating on the scutellum surface are especially useful for biolistic gene transfer experiments (Viertel *et al.,* 1998). Callus formation capacity and plant regeneration depend not only on the type and age of the explants (Özgen *et al.,* 1998), but also on the genotype (Carman *et al.*, 1987; Fennell *et al.,* 1996; Özgen *et al.,* 1998; Dağüstü, 2008), the culture medium (Carman *et al.*, 1987; Fennell *et al.,* 1996; Barro *et al.,* 1999; Rasco-Gaunt *et al.,* 2001), the plant growth regulators (PGRs) and carbon resource concentrations (Mathias, 1990; Rasco-Gaunt *et al.,* 2001; Almouslem *et al.,* 2005) and the culture conditions (He *et al.,* 1988). The developmental stage of immature zygotic embryos plays an important role in the induction of embryogenic callus (Sears and Deckard, 1982; Maddock *et al.,* 1983; He *et al.,* 1988; Vasil, 1987; Özgen *et al.,* 1998; Rasco-Gaunt *et al.,* 2001). Very young or very old immature embryos usually do not form calli or do so only with low efficiency. Particularly with increasing age, immature embryos tend to germinate precociously (He *et al.,* 1988), which is problematic for the subsequent cultivation. Development of a standardized protocol is influenced by the genotype, the age of the immature embryos and the supply of PGRs to the medium. Addition of the artificial auxin 2,4-dichlorophenoxyacetic acid (2,4-D) is indispensable for the induction of scutellar callus (Scott *et al.,* 1990). Concentrations between 1 (Sears and Deckard, 1982; Dağüstü, 2008) and 2 mg/l 2,4-D (He *et al.,* 1988; Redway *et al.,* 1990; Özgen *et al.,* 1998; Varshney and Altpeter, 2001) have been used for callus induction.

The objective of this work was to improve the vegetative propagation of wheat plants from immature embryos as a basis for genetic transformation. To this end, two steps were investigated in detail: the induction and propagation of scutellar calli and the regeneration of wheat plants from those calli. For callus induction and propagation, in addition to different genotypes, two developmental stages of immature zygotic embryos, and two concentrations of 2,4-D were used. The combination of both variables resulted in four different culture conditions. For a reliable statistical analysis, 22 commercial wheat genotypes from three different breeders were used. The varieties investigated are grown in Argentina, which is one of the main wheat exporting countries (FAO 2012).

MATERIALS AND METHODS

Seeds of 22 Argentine spring wheat cultivars were kindly supplied by three breeders from Buenos Aires province: José Buck S.A. (José Buck), Criadores Klein S.A. (Criadores Klein) and Instituto Nacional de Tecnología Agropecuaria (INTA) (Table 1). Donor plants were grown from these seeds in the greenhouse at 20°C and additional illumination was provided during the winter by mercury discharge lamps (180 µmol $m^{-2}s^{-1}$) to maintain a 16-h photoperiod. The substrate was 70 % compost and 30 % clay.

The developmental stage of immature zygotic embryos was determined from morphological features of the grains according to the Zadoks scale (Zadoks *et al.,* 1974). Grains were surface-sterilized in 25 % (v/v) commercial bleach [10.5 % (v/v) NaOCl; 0.3 % (w/v) Na_2CO_3; 10 % (w/v) NaCl; 0.5 % (w/v) NaOH] for 15 min, followed by four washes with sterile distilled water. Immature embryos were aseptically excised under a stereomicroscope in a laminar flow hood and transferred to sterilized callus induction medium. Two developmental stages of immature zygotic embryos (H and W) were identified by two traits: scutellum and axis of the embryo (Table 2). Developmental stage H corresponded to stage II, whereas stage W corresponded to stage IV, as characterized by He *et al.* (1986) (Fig. 1).

ML3 basal medium (Viertel and Hess, 1996), consisting of L3 medium (Jähne *et al.,* 1991) supplemented with 3 %

(w/v) maltose and two concentrations of 2,4-D (1 or 2 mg/l, respectively), was used for induction and maintenance of the calli (callus induction medium). The medium was solidified with 0.2 % Gelrite. The combination of the two developmental stages of immature embryos and two concentrations of 2,4-D resulted in four culture conditions (A, B, C and D) (Table 2). Twenty-five immature zygotic embryos of each variety and treatment were cultured in petri-dishes (90 x 15 mm) with the scutellum up and the embryo axis kept in contact with the solidified callus induction medium (30 ml). This procedure was repeated five times. After three weeks of incubation at 25°C in total darkness, embryogenic scutellar callus formation, callus proliferation on the scutellum and precocious germination were evaluated in all embryos, under a stereomicroscope.

For regeneration of plants, embryogenic calli were transferred to petri dishes containing modified MSB medium (Viertel and Hess, 1996), consisting of MS salts and B5 vitamins (Gamborg *et al.*, 1968), supplemented with 0.5 mg/l 6-benzylaminopurine (BA) and 0.05 mg/l 1-naphthalene acetic acid (NAA) (Ahuja *et al.,* 1982) and solidified with 0.2 % Gelrite.

Cultivation was at 25°C under 16/8h light/dark periods (ca. 54 µmol $m^{-2}s^{-1}$). Plant regeneration capacity and number of regenerated plants were evaluated after 3-4 weeks under a stereomicroscope. To stimulate root growth, plants were transferred to the root-promoting 190-2 medium, as described by Viertel and Hess (1996) without plant growth regulators, supplemented with 3 % (w/v) sucrose, 1 % (w/v) activated charcoal and 0.2 % Gelrite instead of agar.

Plants with well-developed root systems were transferred into autoclaved soil (see above). After acclimation in a culture room, cultivation was continued in the greenhouse (Viertel and Hess, 1996).

Data were analyzed by a chi-square test of independence (contingency table analysis), determination of variance analysis, standard deviation and correlation. If necessary, the *post hoc* test was carried out between the samples from the four culture conditions (A vs. B; C vs. D; A vs. C and B vs. D). Subsequently, the significance calculated was compared with the manually corrected value (Bonferroni-Correction according to Holm). The statistical software package used was SPSS 13.0 (SPSS 2004).

Table 1. Plant breeders and cultivars of the 22 Argentine spring wheat (*Triticum aestivum* L.) genotypes.

Cultivar	Plant breeder
Buck Charrua (BCh)	José Buck
Buck Poncho (BP)	
Klein Cobre (KC)	Criadores Klein
Klein Pegaso (KP)	
Klein Cacique (KCa)	
Klein D. Enrique (KDE)	
Klein Brujo (KB)	
Klein Escorpión (KE)	
Klein Volcán (KV)	
Klein Dragón (KD)	
Klein Estrella (KEs)	
Prointa Oasis (PO)	INTA
Prointa Elite (PE)	
Don Ernesto Inta (DEI)	
Prointa Imperial (PI)	
Prointa Alazán (PA)	
Prointa Granar (PG)	
Prointa Federal (PF)	
Inta Huenpan (IH)	
Prointa Puntal (PP)	
Prointa B. Remodon (PBR)	
Prointa Cauquén (PC)	

Table 2. Experimental treatments. The combinations of the developmental stages of immature zygotic embryos with the two 2,4-D concentrations assayed were identified with the letters A to D.

Stage	Length of embryo (mm)	Length of scutellum (mm) / Appearance	2,4-D (mg/l)	
			1 mg/l	2 mg/l
H	≅ 1.54	≅ 1.30 / Transparent	A	C
W	≅ 2.23	≅ 2.00 / Opaque	B	D

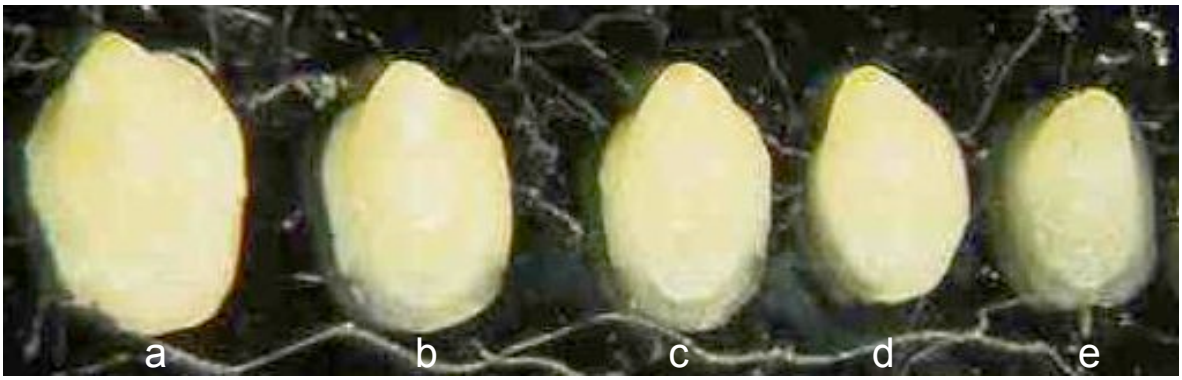

Figure 1. Five different developmental stages of immature zygotic embryos of wheat. Letters a and b represent stage W and letters c-e represent stage H, according to He *et al.* (1986).

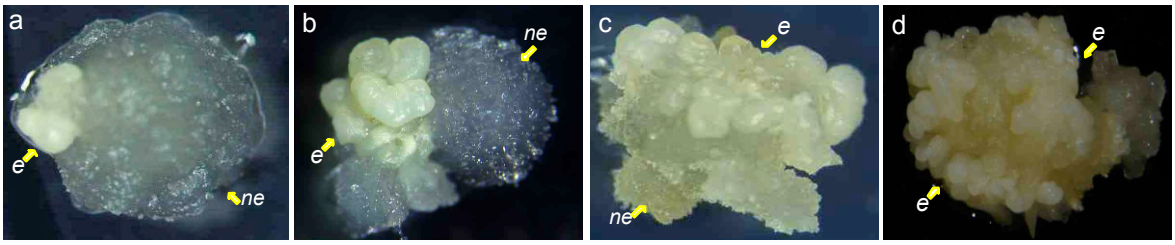

Figure 2. Scutellar callus three weeks after induction of immature embryos. Callus showing embryogenic (e) and nonembryogenic (ne) structures. Estimated percentage of callus proliferation on the scutellum surface: a) 20%; b) 45%; c) 70% and d) 100%.

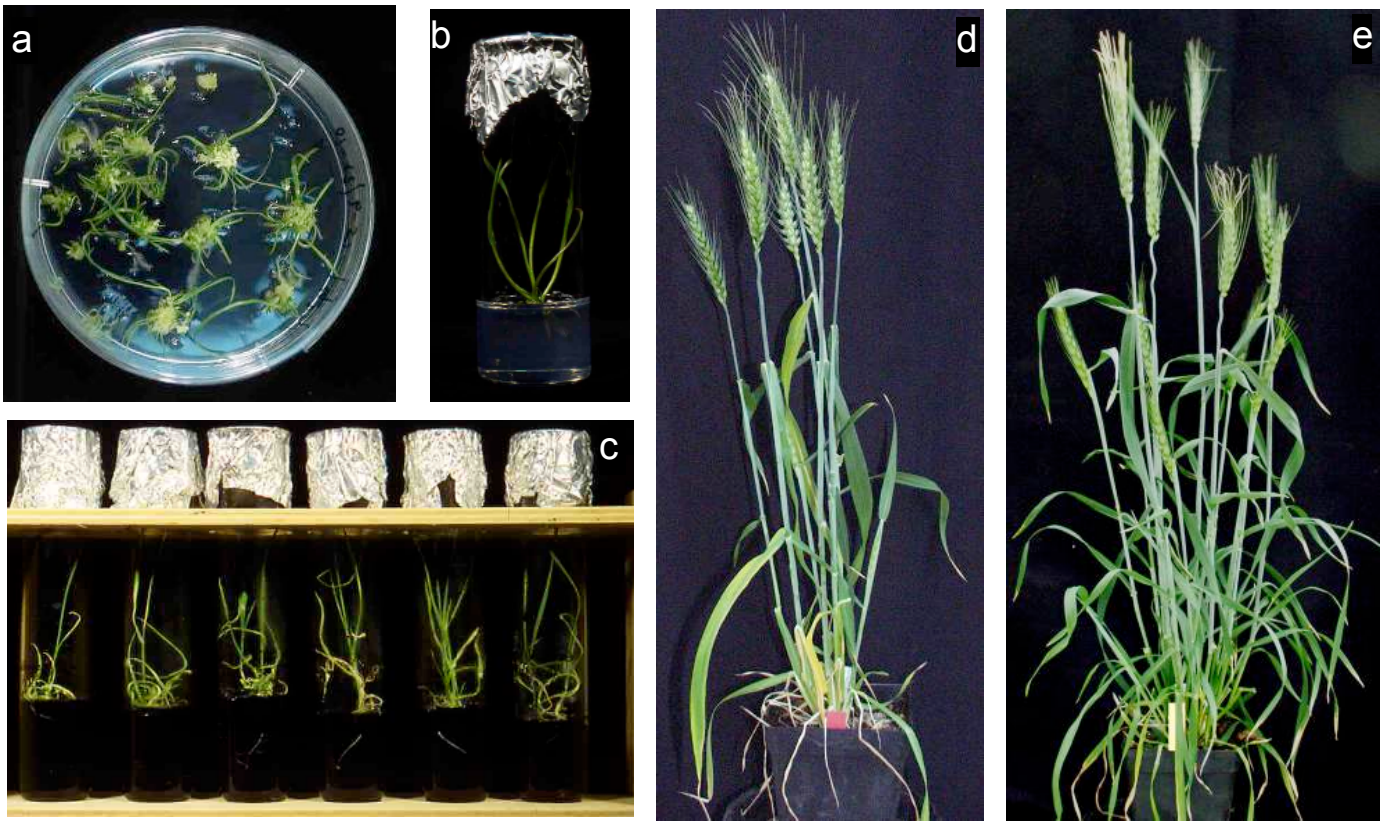

Figure 3. *In vitro* regeneration of wheat: (a) Regeneration on MSB-medium, (b) Continuation of the regeneration in small glass (c) Root formation in 190-2 medium. Greenhouse phase: (d) Mature plant derived from embryogenic scutellar callus and (e) seed-derived control plant of the cultivar *Klein Brujo.*

RESULTS

Induction of scutellar callus

A total of 10091 immature wheat embryos were used as starting material. Explants were able to produce both embryogenic and non-embryogenic calli. Embryogenic calli were nodular and solid, with a white to pale yellow surface, whereas non-embryogenic calli were soft, watery, and translucent (Fig. 2). After 3 weeks of culture on induction medium, all the spring wheat cultivars used, except *Prointa Elite,* produced embryogenic scutellar calli. Induction of scutellar embryogenic calli was significantly dependent on the culture conditions (Chi-square-test X^2 = 1017.5; p<0.001), as revealed by the rates of callus formation. The capacity of embryogenic callus formation varied widely among genotypes, ranging from 20.5 % to 85.7 % under culture condition A, from 0.0 % (five genotypes) to 46.8 % under culture condition B, from 10.1 % to 86.4 % under culture condition C, and from 0.0 % (two genotypes) to 89.3 % under culture condition D (Table 3). Under culture condition A, production of embryogenic calli was higher than 75 % with five genotypes (*Klein Brujo, Klein Pegaso, Klein Dragón, Prointa Puntual, Prointa Federal*), under culture condition C, with two of the genotypes (*Klein Dragón, Prointa Imperial*), and under condition D with one genotype (*Prointa Federal*). Under culture condition B, none of the genotypes reached 50% (Table 3).

Other factors influencing the formation of embryogenic calli from immature embryos were the stage of development and the concentration of 2,4-D. Immature donor embryos of developmental stage H and 1 mg 2,4-D/l medium (culture condition A) produced the best results, whereas embryos of the same stage and 2 mg 2,4-D (culture condition C) produced the second best results, being the difference between the two conditions statistically significant (Table 3). In contrast, increasing the concentration of 2,4-D from 1 to 2 mg/l medium had consistently positive effects when immature donor embryos of stage W were used (Table 3).

Capacity of callus proliferation of the cells of the scutellum surface

Callus proliferation of the cells of the scutellum surface was estimated and given in percent of the total scutellum surface (Fig. 2). The capacity of scutellum surface cells to form calli varied with the culture conditions and the genotype: from 30.0 % to 84.4 % under culture condition A, from 0.0 % to 63.3 % in culture condition B, from 25.0 % to 71.2 % in culture condition C, and from 25.0 % to 67.0 % in culture condition D (Table 3). The differences between the individual conditions were highly significant (Chi-square-test X^2 = 16.5; p<0.01). However, callus proliferation capacity varied less with the culture conditions than callus formation capacity as such (Table 3). The developmental stage of the immature embryos, however, was more crucial, being stage H significantly better than stage W (Table 3). On the other hand, the concentration of 2,4-D was less important for callus proliferation. Like the capacity of scutellar embryogenic callus formation, culture condition A produced the highest callus proliferation (two genotypes above 75%, *Klein Brujo, Klein Dragón*, and 11 above 50%), whereas culture condition C produced the second highest callus proliferation (11 genotypes above 50%, but none above 75%). A significant correlation between scutellar embryogenic callus formation and callus proliferation on the scutellum surface was observed under culture conditions A (r = 0.56; p = 0.006) and C (r = 0.47; p = 0.030).

Precocious germination of immature embryos

Precocious germination of the immature zygotic donor embryos varied among genotypes, ranging from 0.0 % to 32.1 % in culture condition A, from 0.0 % to 78.4 % in culture condition B, from 0.0 % to 31.4 % in culture condition C, and from 0.0 % to 97.7 % in culture condition D (Table 3). Even the otherwise not responding immature embryos of *Prointa Elite* showed precocious germination. Most failures of precocious germination were observed under the conditions C (11 out of 22 genotypes) and D (5 out of 22 genotypes) (Table 3). Both the culture conditions (Chi-square-test X^2 = 985.7; p<0.001) and the genotype influenced the extent of precocious germination (Table 3). Developmental stage H and 2 mg 2,4-D/l medium (culture condition C) was optimal for the inhibition of precocious germination. Differences with the other conditions were highly significant (Table 3). The frequency of precocious germination was highest in culture condition B.

Plant regeneration

The potential of scutellar embryogenic calli to regenerate whole plants after transfer to modified MSB medium (Fig. 3a and b) was investigated. Plant regeneration from scutellar embryogenic calli ranged from 46.4 % to 100 % under culture condition A, from 0.0 % to 100 % under culture condition B, from 30.0 % to 95.2 % in culture condition C, and from 0.0 % to 100 % in culture condition D (Table 4). Remarkably, under conditions A and C, all genotypes (but not all calli) produced plants. The composition of the callus induction medium and the developmental stage of the immature donor embryos influenced plant regeneration (Chi-square-test X^2 = 139.8; p<0.001). As expected, the genotype was also important. Culture condition A, i.e. immature embryos of stage H in a medium with 1 mg 2,4-D/l, was the best combination for the subsequent regeneration of plants (14 out of 21 = 66.6 %), which was triggered by transfer of the calli to regeneration medium. Developmental stage W in the same induction medium (culture condition B, 3 out of 21 = 14.3 %), as well as stage H cultivated in induction medium with 2 mg 2,4-D/l (culture condition C, 4 out of 21 = 19.0 %) were significantly less efficient (Table 4). For culture condition A, 20 genotypes showed regeneration efficiency over 50%, three of which (*Klein Brujo, Inta Huenpan,* and *Prointa B. Remodon*) reached 100%. For culture condition C, 19 genotypes showed regeneration efficiency over 50%, but none reached 100 % (Table 4). We found no relationship between scutellar embryogenic callus induction and plant regeneration capacity of the callus. We observed that genotypes exhibiting relatively poor callus induction frequency did not necessarily produce fewer regenerable calli (Table 3 and 4).

Table 3. Effect of the combination of the two developmental stages of immature zygotic embryos with the two 2,4-D concentrations (culture conditions A, B, C and D) on the frequency of scutellar callus induction, callus proliferation on the scutellum and precocious germination from twenty two Argentine wheat genotypes.

Cultivar	Scutellar callus induction (formation) (%)[1, 3]				Callus proliferation on the scutellum (%)[4]				Precocious germination (%)[2,5]			
	A	B	C	D	A	B	C	D	A	B	C	D
BCh.	62.0	46.8	48.3	35.7	39.0	34.0	48.0	33.3	0	0	0	31.0
BP.	59.7	0	43.6	9.4	55.0	0	49.0	45.0	6.3	66.0	0	5.7
KC.	20.5	0	10.1	8.6	31.0	0	38.9	35.0	23.8	27.5	0	6.9
KP.	84.7	19.1	48.8	10.7	50.0	34.1	50.6	30.0	3.4	27.0	0	23.2
KCa.	19.8	23.2	40.7	11.8	52.0	38.0	43.0	39.0	14.8	25.6	0	7.9
KDE.	38.5	32	18.2	26.4	52.0	41.1	55.0	32.1	4.8	9.6	0	15.1
KB.	84.8	33.6	72.5	35.9	77.0	40.4	71.2	48.2	0.6	36.7	2.5	25.0
KE.	31.4	0	45.5	2.8	30.0	0	25.0	25.0	23.5	72.1	9.1	86.1
KV.	68.3	8.8	63.3	15.4	52.1	33.3	63.3	33.3	8.1	34.1	0	7.7
KD.	85.7	31.3	76.1	29.4	84.4	58.9	67.9	49.1	5.9	29.0	0	29.4
KEs.	64.6	22.1	61.5	1.8	53.9	45.6	63.5	37.5	9.5	37.1	5.1	43.0
PO.	27.7	7.1	58	4.7	59.6	31.3	56.0	37.5	4.2	48.2	5.8	48.8
PE.	0	0	0	0	0	0	0	0	32.1	37.2	31.4	23.3
DEI.	57.1	13.3	55.6	53.7	63.3	52.9	69.3	54.5	21.4	65.6	0	0
PI.	53.2	0	86.4	0	38.0	0	39.5	0	19.2	50.0	0	0
PA.	33.8	28.6	29.6	0	42.9	62.5	43.8	0	21.5	0	11.1	0
PG.	58.7	16.7	66.7	53.2	55.6	63.3	57.2	67.0	7.7	14.0	4.4	0
PF.	78.3	5.0	58.6	89.3	46.9	37.5	56.5	37.9	2.4	67.5	0	2.9
IH.	32.6	11.1	13.2	10	50.0	43.7	35.7	31.3	2.3	29.2	9.4	0
PP.	80.4	14.9	72.6	1.2	65.4	40.9	50.9	50.0	23.9	78.4	26.8	97.7
PBR.	46.2	2.9	64.5	25	40.0	33.3	47.7	43.8	8.8	28.4	12.9	37.5
PC.	31.5	0	30.8	10.5	30.0	0	39.6	25.0	6.9	67.5	12.8	36.8
Means ± SD	51.0 ± 24.4^{ae}	14.4 ± 13.7^{bg}	48.4 ± 23.2^{cf}	19.8 ± 22.6^{dh}	48.6 ± 17.7^{ae}	31.4 ± 21.6^{bf}	48.7 ± 16.0^{ce}	34.3 ± 17.1^{df}	11.4 ± 9.4^{ae}	38.7 ± 23.1^{bg}	6.0 ± 8.8^{cf}	24.0 ± 26.9^{dh}

[1] Number of immature embryos forming embryogenic callus / number of immature embryos X 100

[2] Number of immature embryos with germinating zygotic embryo / number of immature embryos X 100

[3 a-h] Means followed by the same letter are not significantly different at 1 % level.

[4 a-f] Means followed by the same letter are not significantly different at 5 % level

[5 a-h] Means followed by the same letter are not significantly different at 0.1 % level

Table 4. Frequency of plant regeneration capacity, culture efficiency and number of plants regenerated per embryo (precultured under condition A, B, C and D) cultured on MSB-medium.

Cultivar	Plant regeneration capacity (%)[1, 4]				Number of plants regenerated[2, 5]				Culture efficiency (%)[3, 6]			
	A	B	C	D	A	B	C	D	A	B	C	D
BCh.	93.3	73.6	93.7	74.2	2.1	1.6	2.7	1.7	57.8	34.4	45.3	26.5
BP.	77.7	-	57.9	55.5	7.9	-	5.6	4.4	46.4	-	25.2	5.2
KC.	61.3	-	57.6	48	5.3	-	5.3	2.9	12.6	-	5.8	4.1
KP.	72.5	60.0	64.7	72.5	2.9	2.6	5.0	1.8	61.4	11.5	31.6	7.8
KCa.	46.4	0	68	12.5	1.8	0	2.0	2.0	9.2	0	27.7	1.5
KDE.	73.3	66.6	68	0	6.1	6	6.4	0	28.2	21.3	12.4	0
KB.	100	100	76.3	100	9.5	7.6	5.6	5.0	84.8	33.6	55.3	35.9
KE.	78.8	-	65	50	7.5	-	6.6	4.6	24.7	-	29.6	1.4
KV.	96.3	100	95.2	100	6.8	4.2	8.3	6.4	65.8	8.8	60.3	15.4
KD.	97.0	74.2	30	42.9	9.5	4.5	1.7	4.8	83.1	23.2	22.8	12.6
KEs.	54.3	8.6	37.9	25.8	5.7	3.3	4.9	4.8	35.1	1.9	23.3	0.5
PO.	94.4	100	72.7	56.3	3.8	3.3	3.4	3.3	26.2	7.1	42.2	2.6
PE.	-	-	-	-	-	-	-	-	-	-	-	-
DEI.	92.3	81.2	91.7	87.1	5.8	2.0	1.6	1.8	52.7	10.8	51.0	46.8
PI.	88.6	-	64	-	3.7	-	3.4	-	47.1		55.3	
PA.	85.7	56.3	88.8	-	2.3	2.0	2.3	-	29.0	16.1	26.3	
PG.	65.7	56.2	66.5	52.9	2.5	1.8	3.6	3.1	38.6	9.4	44.4	28.1
PF.	93.1	0	83.3	66.6	5.6	0	2.3	3.2	72.9	0	48.8	59.5
IH.	100	65.5	75	57.1	2.3	3.1	2.0	2.2	32.6	7.3	9.9	5.7
PP.	85.5	95.2	83.9	80.6	8.0	7.4	7.6	6.0	68.7	14.2	60.9	1.0
PBR.	100	82.3	93.5	80	2.9	6.0	2.1	2.4	46.2	2.4	60.3	20.0
PC.	92.3	-	79.1	66.6	4.2	-	3.4	2.4	29.1		24.4	7.0
Means ± SD	83.3 ± 15.8^{ae}	63.7 ± 33.8^{bf}	72.0 ± 17.4^{ce}	59.4 ± 26.7^{df}	5.1 ± 2.5^{ab}	3.5 ± 2.3^{ac}	4.1 ± 2.0^{ab}	3.3 ± 1.7^{ac}	45.3 ± 21.8^{ae}	12.6 ± 10.8^{bf}	36.3 ± 17.4^{ce}	14.8 ± 17.2^{dg}

[1] Number of regenerable calli / number of calli induced X 100.
[2] Number of regenerants per embryo cultured gave as mean numbers
[3] Number of regenerable calli / number of embryos cultured X 100 (regenerable callus = nodular callus with green spots).
[4 a-f] Means followed by the same letter are not significantly different at 0.1 % level
[5 a-c] Means followed by the same letter are not significantly different at 5 % level
[6 a-g] Means followed by the same letter are not significantly different at 1 % level

Number of plants arising from scutellar embryogenic callus

Because usually more than one embryogenic callus was formed on the surface of the scutellum, differing numbers of plants were expected from one immature embryo (Fig. 3a). Effects of the genotype as well as of the culture conditions during scutellar callus induction were investigated. Culture condition A (1.8 – 9.5, average 5.1 plants per donor embryo) again proved better than the other three conditions, which gave rather similar results (B: 1.6 – 7.6, average 3.5; C: 1.6 – 7.6, average 4.1; D: 1.7 – 6.4, average 3.3) (Table 4). However, the differences were not statistically significant (Kruskal-Wallis-test: $X^2 = 57.4$ $p = 7.3$). The number of plants regenerated and the plant regeneration capacity of scutellar embryogenic calli were not significantly correlated.

Because of the high number of plants, plant samples were taken randomly from each cultivar and culture condition. Each plant regenerated was transferred into soil in greenhouse conditions (*ex vitro*) after *in vitro* root promotion (Fig. 3c). In that phase, neither the cultivar nor the culture condition influenced the morphogenetic development (Fig. 3d). All plants matured normally and set fertile seeds after approximately three months.

Overall efficiency of the four culture conditions

Given the wide range of embryogenic callus formation displayed by the 22 cultivars investigated and their differing capabilities for plant regeneration, the significant differences in overall efficiencies in plant regeneration were not unexpected (Chi-square-test $X^2 = 31.9$; $p<0.001$). Comparison of the influence of culture conditions A – D showed that younger immature donor embryos (stage H) were better than more advanced ones (stage W), irrespective of the concentration of 2,4-D in the medium (Table 4). With donor embryos of stage H, the lower concentration of 2,4-D (1 mg 2,4-D/l) was better than the higher one (2 mg 2,4-D/l), whereas with donor embryos of stage W, the higher concentration was slightly more effective. The influence of the genotype was overridden by the culture conditions, i.e. the values of overall efficiency displayed by the 22 cultivars were not identical after the four culture conditions, not even when the same developmental stage H or W was considered: while *Klein Brujo* (84.8 %), *Klein Dragón* (83.1 %) and *Prointa Federal* (72.9 %) were the best after preculture under condition A, *Prointa Puntal* (60.9 %), *Prointa B. Remodon* (60.3 %) and *Klein Volcán* (60.3 %) were the best after preculture under condition C. When using developmental stage W, the three best varieties were *Prointa Federal* (59.5 %), *Don Ernesto Inta* (46.8 %) and *Klein Brujo* (35.9 %) after preculture under condition D, and *Buck Charrua* (34.4 %), *Klein Brujo* (33.6 %) and *Klein Dragón* (23.2 %) after preculture under condition B.

In decreasing order, the four cultivars with highest scutellar embryogenic callus were *Prointa Federal* (89.3 %) under culture condition D, *Prointa Imperial* (86.4 %) under culture condition C, and *Klein Dragón* (85.7 %) and *Klein Brujo* (84.8 %) under culture condition A. Nevertheless, this order changed when the culture efficiency and the number of plants regenerated were considered. The first place was occupied by *Klein Brujo* with 84.8 % and 9.5 respectively, followed by *Klein Dragón* (83.1 %, 9.5), *Prointa Federal* (59.5 %, 3.2) and *Prointa Imperial* (55.3 %, 3.4).

We may conclude that among the 22 spring wheat varieties investigated, *Klein Brujo* was the best when pre-cultured under condition A, but also yielded moderate to good results under the other three conditions. *Klein Dragón* was the second best, with similar good results under condition A but not under the other three conditions.

DISCUSSION

Variables and their interactions in callus formation and plant regeneration

The data presented show that induction of scutellar callus, precocious germination of immature embryos, capacity of scutellar embryogenic callus to grow into plants and the entire culture efficiency, all depended on the genotype, the stage of development of the immature embryo, the concentration of 2,4-D in the medium, and the interaction(s) between them. While the capacity of scutellar calli to develop new shoots under the experimental conditions applied depended only on the genotype, callus proliferation on the scutellum also depended on the developmental stage of the immature embryos.

The significant influence of the genotype on both processes has also been observed in other studies (Carman *et al.*, 1987; Fennell *et al.,* 1996; Özgen *et al.,* 1998; Viertel *et al.,* 1998; Barro *et al.,* 1999; Dağüstü, 2008). In this work, the importance of the developmental stage was given special attention in combination with the concentration of the hormone in the medium. In agreement with that reported by León *et al.* (2006), the younger stage of the immature embryo was more effective and the effect of maturation of the embryo could not be counteracted by

doubling the 2,4-D concentration of the medium. The higher 2,4-D concentration was more effective in the promotion of callus formation only if older embryos (stage W) were used, a finding which is line with that reported by Almouslem *et al.* (2005). Using young immature embryos (stage H), the higher concentration of the auxin in the medium usually inhibited both morphogenetic processes, as well as the precocious germination of the embryo. This finding was conflicting because callus formation and propagation and regeneration of plants from the calli were more effective with the lower concentration of auxin in the medium, which also promoted precocious germination. Either a slight or no inhibitory effect of a higher 2,4-D concentration on embryogenic callus formation and plant regeneration was reported by Viertel *et al.* (1998) for 18 German spring wheat varieties. In agreement with our results, Barro *et al.* (1999) found that the concentration of 2,4-D played a crucial role and that the lower concentration was better for scutellar embryogenic callus formation and regeneration. A loss of responsiveness to the hormone with the advance of maturation of the embryo cannot be ruled out as an explanation of the phenomena observed. Inhibition of the increase in precocious germination as an indicator of the advancing maturation required higher auxin concentration in the medium.

Is there a correlation between the potentials of callus induction and plant regeneration from the calli?

Vegetative propagation of wheat varieties by tissue culture methods depends on both a high potential for embryogenic callus production and a high capacity of plant regeneration from the calli. In the Argentine spring wheat cultivars studied, the regeneration rate of plants from calli was high, but the scutellar callus formation rate of the immature embryos was rather low. Not the entire surface of the wheat-scutellum is capable of forming embryogenic callus (Scott *et al.,* 1990). In that respect, callus proliferation on the scutellum surface is an important parameter for biolistic transformation of wheat. However, only Viertel *et al.* (1998) studied this issue. These authors found an increase in the portion of the callus producing scutellar surface when 1 mg/l instead of 2 mg/l of 2,4-D was used in the medium. Like in our present work, Viertel *et al.* (1998) also reported that there was a positive correlation among the frequency of immature embryos forming embryogenic callus and the callus proliferation on the scutellum surface. In the present study, with a substantial collection of Argentine spring wheat cultivars, the young developmental stage of the immature embryo produced a higher percentage of callus proliferation, although the variation between the genotypes was considerable.

Even with the best of our protocols (culture condition A), the overall efficiency of the entire tissue culture approach did not exceed the values reported for South American wheat cultivars (Chowdhury *et al.,* 1991).

In wheat, like in other plants, the variation in tissue culture response is assumed to be controlled by the interaction of a large number of genes (polygenes) (Fennell *et al.,* 1996; Varshney and Altpeter, 2001). With the 22 Argentine spring wheat varieties investigated in this work, we found no relationship between embryogenic callus formation and regeneration capacity. This is in agreement with observations by Sears and Deckard (1982), Chowdhury *et al.* (1991) and Özgen *et al.* (1996, 1998). It appears that both phenomena are controlled by different genes or gene combinations. However, Viertel *et al.* (1998), Barro *et al.* (1999) and Dağüstü (2008) reported a close correlation between both processes and, at the current state of knowledge, there is no explanation for this contradiction.

Reports about the numbers of plants regenerated per cultured explant differ significantly, a fact that may be explained by the genotypic variation and the culture conditions. In the Argentine spring wheat cultivars studied, the influence of the culture conditions appeared to be restricted to the early stages of the tissue culture protocol. Once shoot regeneration from calli was induced, neither the developmental stage of the immature embryo nor the auxin concentration in the medium played a particular role in the number of plants regenerated.

The Argentine wheat genotypes analyzed regenerated a low number of plants per embryo (1.6 to 9.5) in comparison with other reports (Fennell *et al.,* 1996: 6 to 42; Viertel *et al.,* 1998: 1 to 19; León *et al.,* 2006: 2.3 to 16.8). Only the genotypes examined by Varshney and Altpeter (2001) showed results (1.01 to 9.2) similar to those of the present study.

The present study aimed to gain knowledge on the best conditions for successful genetic transformation of spring wheat. Embryogenic scutellar calli are the most widely used target tissue for gene transfer in wheat. As the medium is important for embryogenesis in these calli, it could also be used as selection medium for transgenic plants. Among the 22 Argentine spring wheat genotypes studied, *Klein Brujo*

and *Klein Dragón* seemed to be the most promising. The present study also showed that standard culture conditions cannot be applied for all genotypes. This result concurs with the hypothesis of Redway *et al.* (1990), which establishes that the differences in genotype response are physiological in nature and thus require adjustment of the *in vitro* culture conditions and media.

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