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Callus induction and plant regeneration by Brazilian new elite wheat genotypes

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ABSTRACT – *The distinction of genotypes responsive to tissue culture and the development of an efficient regeneration system are the first steps towards transgenic plant production. Nine Brazilian wheat (*Triticum aestivum *L.) genotypes were cultivated in vitro to evaluate the embryogenetic capacity. The explants (immature zygotic embryos) were tested in two different culture media, MS (Murashige and Skoog 1962) and modified MS - MMS (Zhou et al. 1995) with decreasing dosages of hormone regulators. Three distinct phases were observed in each medium: induction, maintenance and regeneration. After induction, the somatic embryogenesis of calli was evaluated every 21 days. Genotypes responded differently to the different culture media. The embryogenic response of genotype CD104 was best in both culture media tested. On MMS, the values of callus induction, plant regeneration and ratio of regenerated plantlets per rescued embryo of this genotype were 100%, 99.5% and 1.1%, respectively. Genotypes CD104, CD200126 and CDFAPA 2001129 were most responsive on MS (regeneration capacity of 37.5%, 33.5% and 33% respectively), and therefore interesting for genetic transformation in plant breeding programs that develop new elite cultivars with a commercial purpose.*

Keywords: wheat, *Triticum aestivum*, callus induction, plant regeneration, culture medium, embryogenesis.

INTRODUCTION

Genetic engineering of any species depends on the development of efficient and reliable callus induction and plant regeneration systems. One of the many limiting factors in wheat breeding is the inferior quality of wheat cultivars available with low performance of agronomic traits (Jones 2005). In wheat transformation protocols, the use of elite wheat cultivars as exogenous gene recipients can speed up the process of commercial field applications of transgenic wheat. However, it is seldom known which elite cultivars have a good level of tissue culture response (TCR).

Several authors have studied TCR of elite wheat cultivars in European (Pastori et al. 2001); Asian (Arzani and Mirodjagh 1999, Wu et al. 2002, Li et al. 2003), Mexican (Fennell et al. 1996); Australian (Witrzens et al. 1998), and Chinese genotypes (Tang et al. 2006). On the other hand, few papers were found in the literature dealing with the regeneration performance of Brazilian genotypes (Dornelles et al. 1997).

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Monocots seem to be less responsive to *in vitro* techniques of regeneration tissue culture, partly due to the fact that in cereals only cells and immature or young tissues can be induced, which inherently respond efficiently to regeneration (Repellin et al. 2001). In wheat, different explant sources were studied: shoot tips (Viertel and Hess 1996); seeds (Gosch-Wackerle et al. 1979); inflorescences (Redway et al. 1990); young leaves (Zamora and Scott 1983); mature embryos (Delporte et al. 2001, Wu et al. 2002); immature embryos (Macchii et al. 1998); and anthers (Brisibe et al. 2000). Immature embryos seem to be the best explant source for callus induction and somatic embryogenesis of cereals (Wu et al. 2002, Pellegrineschi et al. 2004).

Several factors can influence the regenerative potential of a specimen: genotype, concentration of different growth regulators, explant types and sizes, culture media and culture conditions (El-Sherbeny et al. 2001, Tamás et al. 2004). Several media for tissue culture used with immature embryos are quoted in literature: MS (Musharige and Skoog 1962) (Harvey et al. 1999, Gonzalez et al. 2001); N6 (Wu et al. 2002); B5 (Vasil and Vasil 1994) and modified MS - MB (Li et al. 2003); P (Delporte et al. 2001); CM4/MMS (Cheng et al. 1997); and DSEM (Eudes et al. 2003), with different combinations of components and growth regulators.

The aim of this paper was to screen Brazilian genotypes of germplasm released by Coodetec in the last years with regard to the ability of callus induction and plant regeneration by somatic embryogenesis through immature embryo culture on two distinct culture media, with a view to the establishment of a suitable regeneration system for these cultivars.

MATERIAL AND METHODS

Nine wheat genotypes (*Triticum aestivum* L.) were evaluated for *in vitro* regeneration capacity: CD104, CD105, CD110, CD200111, CD113, CD200117, CD105, CD200126, and CDFAPA 2001129, originated from the wheat breeding program developed by the Central Cooperative of Agricultural Research (Coodetec), Cascavel, Brazil.

Stock plants were grown in a greenhouse under day and night temperatures of around 25°C ± 2 and 18°C ± 2, respectively. The explant source consisted of immature embryos (about 0.5-1.5 mm long), collected from seeds in the milky phase, approximately 14-16 days after anthesis. The seeds were disinfected with a commercial solution of 2.5% sodium hypochlorite for 20-30 min, followed by three rinses in sterile water. The seeds were then immersed in 70% (v/v) ethanol for 1 min and dried on filter paper. The immature embryos were aseptically excised from caryopses and placed scutellum upwards on induction medium.

Two induction media were tested: MS (Murashige and Skoog 1962), with 2.0 mg L^{-1} 2,4-D, solidified with 3.0 g L^{-1} Phytagel™, and MMS (Zhou et al. 1995) composed of MS macro and micronutrients, supplemented with 2.2 mg L^{-1} Picloram (4-amino-3,5,6-trichloropicolinic acid) (Padron/DOW™); 0.5 g L^{-1} glutamine; 0.75g L^{-1} magnesium chloride; 0.1g L^{-1} peptone; 1.95 g L^{-1} MES; 0.5 mg L^{-1} 2,4-D; 30 g L^{-1} sucrose; 100 mg L^{-1} ascorbic acid solidified with 3.0 g L^{-1} Gelrite™. The media were adjusted to pH 5.8 before sterilization.

The callus maintenance media were: MS with concentrations of 1.0 mg L^{-1} 2,4-D solidified with 3.0 g L^{-1} of Phytagel™, and the MMS supplemented with 0.2 mg L^{-1} 2,4-D solidified with 3.0 g L^{-1} Gelrite™. The regeneration media consisted of MS macro and micronutrients supplemented with 1.95 g L^{-1} MES; 30 g L^{-1} sucrose; 100 mg L^{-1} ascorbic acid, while no growth regulators were added to either of the media.

Ten embryos were cultured on each Petri dish (8.5 cm diameter), using 20 plates per genotype per culture medium (MS and MMS). The temperature in the growth chamber was maintained at around 25°C during the entire culture period. During the induction phase, embryos were kept in darkness during 10 days and then exposed to light for 11 days. After this period, calli were transferred to the maintenance medium and incubated for 21 days, then to regeneration medium for 15 days, and finally transferred to the maintenance media. During the maintenance and regeneration phases, the photoperiod cycle for calli and somatic embryos was 16-h light: 8-h dark, at a light intensity of 30-mmol m^{-2} s^{-1} under white fluorescent lamps.

Statistical Analysis: Data were analyzed by ANOVA, in a complete randomized design, considering each dish as one replicate of each treatment. Treatments were grouped by the Scott Knott test (Scott and Knott 1974) using the statistical software Genes (Cruz 1997). The percentage of callus induction, regeneration capacity and the proportion of induced plantlets and calli were also evaluated. The percentage of induced

was calculated by the ratio between the number of induced calli based on immature embryos and the number of rescued per genotype after 21 days on induction medium. The regeneration capacity of each genotype was determined by the percentage of embryogenic calli with green spots. The ratio between number of plantlets obtained and number of induced calli was also evaluated. Plantlets were only included in the count when complete structures were formed and regeneration from embryogenic calli had started, considering plantlets as complete when leaves and roots were clearly formed.

RESULTS AND DISCUSSION

Callus induction and growth from immature embryos was visible in both media and in all genotypes after 6-10 days in the dark. The calli induced were soft, white and friable. After 72 days of culture the embryogenic calli were transferred to regeneration medium to grow the plantlets.

A two-way ANOVA was used to analyze the effects of genotype and culture medium as well as their interaction on the number of regenerated plantlets. The levels of callus induction and plant regeneration were satisfactory in almost all genotypes but differences were observed between genotypes and culture medium, and interaction between genotypes and culture medium. On the other hand, not all genotypes regenerated plantlets in both media.

The performance of the nine genotypes evaluated in two distinct *in vitro* media (MS and MMS) differed for the parameters analyzed by the Scott Knott test (Table 1). On the MS medium, callus induction was similar among all genotypes. The highest number of calli was observed in CD104 and CDFAPA 2001129 (mean values of 8.40±1.79 and 7.05±0.65 calli per Petri dish, respectively). However, on MMS medium, genotypes responded differently. The highest capacity of callus induction was observed in CDFAPA2001129; CD113; CD200117 and CD200126 (9.05±1.00; 8.75±1.54; 8.30±1.30 and 8.05±1.34, respectively). Scores were lowest for genotypes CD 110 and CD 111.

The best performance regarding number of regenerated plantlets (NRP) on MMS medium was observed in CD104, with an average of 8.44±3.37 regenerated plantlets. On MS medium, the best performance was observed in CD200111, with an average of 13.16±1.23 regenerated (Table 1).

The range between number of calli and number of rescued immature embryos of the tested genotypes varied from 65.5% to 100% and the regeneration capacity from 0 to 99.5% (Table 2). For induction response, results in the literature range from 92-100% (Ozgen et al. 1996), 20-100% (Machii et al. 1998) and for regeneration capacity, from 29 -100% (Maddock et al. 1983); 0-60% (Mathias and Simpson 1986); 0-90% (Machii et al. 1998); and 28-60% (Li et al. 2003).

Table1. Performance of nine Brazilian wheat genotypes in terms of callus induction and regenerated plantlets in two different culture media

Genotype	NC		NRP	
	MMS	MS	MMS	MS
CD104	7.39±1.75^{b1}	8.40±1.79^{a}	8.44±3.37^{a}	7.53±2.78^{b}
CD111	6.75±1.79^{c}	6.60±1.82^{a}	1.30±0.72^{d}	4.87±1.77^{c}
CD110	6.65±1.29^{c}	7.25±1.52^{a}	0.0±0.0^{d}	0.0±0.03^{d}
CD200111	7.75±1.61^{b}	7.25±1.56^{a}	0.0±0.0^{d}	4.66±0.97^{c}
CD113	8.75±1.54^{a}	7.25±1.52^{a}	6.5±0.71^{b}	0.0±0.0^{d}
CD200117	8.30±1.30^{a}	6.15±1.52^{a}	5.0±0.70^{b}	13.16±1.23^{a}
CD105	7.65±1.22^{b}	6.85±1.19^{a}	3.83±0.94^{c}	1.0±0.36^{d}
CD200126	8.05±1.34^{a}	6.95±1.30^{a}	2.93±1.02^{c}	8.95±1.45^{b}
CDFAPA 2001129	9.05±1.00^{a}	7.05±0.65^{a}	1.86±0.54^{c}	7.95±1.95^{b}

[1]Means followed by the same letter in the same column did not differ at P=0.05 by the Scott and Knott (1974) test.
MMS = Modified Murashige and Skoog medium
MS = Murashige and Skoog medium
NC = Number of calli
NRP = Number of regenerated plantlets

The induction capacity of genotypes CDFAPA 2001129 on MS and CD104 on MMS was best (88% and 100%, respectively). The genotypes CD104, CDFAPA 2001129 and CD200126 were distinguished on MS with the highest regeneration index (37.5%, 33.5% and 33 %, respectively). On MMS, genotype CD104 was most responsive to regeneration (99.5%). The values regarding the number of plantlets obtained from embryogenic calli for each genotype indicated cultivars CD104, CD200126 and CD200117 as most responsive to somatic embryogenesis (Table2).

Although the values for induction capacity in both media were similar for genotype CD111 (67.5% and 66% on MMS and MS, respectively), the values were contrasting in relation to regeneration capacity: the regeneration capacity or competence was 18.2% on MS and 7.4% on MMS medium. With regard to the regenerative capacity, similar results - the highest genotype induction capacity was not associated to the best results - were obtained by Benkirane et al. (2000) in durum wheat (*Triticum durum*) using inflorescence culture. On the other hand, Ozgen et al. (1996) observed a positive and significant correlation between the high frequency of callus induction and number of regenerated plants. A possible explanation for this correlation would be the genotypic difference for the formation of more non-embryogenic than of embryogenic calli (green spots), which is directly related to the regeneration capacity.

The mean values of genotype CD110 were 6.65± 1.29 and 7.25±1.52 calli per dish (MMS and MS media, respectively), corresponding to 70% callus induction, whereas no regenerative capacity was observed in either of the culture media.

The influence of auxins on wheat regeneration was genotype-dependent (Przetakiewcz et al. 2003). Since the cited paper aimed at establishing an efficient and quickly reproducible tissue culture protocol only two media were compared without testing different matching levels of growth regulators, but only the presence and absence of the auxins 2,4-D and Picloram. In our study, the response of some genotypes to callus induction and regeneration was better, probably due to the presence of additional components in the MMS medium (glutamine, magnesium chloride; peptone; MES, and ascorbic acid). This was the case for CD113, which grew on MMS only, as well as for CD104 and CD105, with higher ratios of number of MMS induced plantlets per callus.

Some authors reported a better response of Picloram compared to 2,4-D for induction and long-term maintenance of regenerative callus from immature embryo explants in barley (Kachhwaha et al.1997), a species related to wheat, and d*urum* wheat (Satyavathi et al. 2004). Barro et al. (1999) reported that in wheat the addition of Picloram (2 mg L^{-1}) together with 2,4-D in culture medium increased plantlet regeneration by 50%.

However, the better performance of other genotypes on MS medium, such as CD200111, CD111, CD200117, CD200126, CDFAPA 200129, besides easier and cheaper, makes the MS medium more interesting for efficient and reproducible wheat TCR. Other authors report the superiority of MS in cereals in terms of callus induction frequency and green spot formation (He et al. 1988, Pellegrineschi et al. 2004).

Table 2. Performance of nine Brazilian wheat genotypes evaluated in relation to percentage of callus induction, percentage of embryogenic capacity and the ratio of plantlets regenerated by induced calli, in two different culture media

Genotypes	Callus induction (%)		Regeneration Capacity (%)		Number of Plantlets Induced Calli^{-1}	
	MMS	MS	MMS	MS	MMS	MS
CD104	100.0	84.0	99.5	37.5	1.1	0.7
CD111	67.5	66.0	7.4	18.2	0.1	0.5
CD110	66.5	72.5	0.0	1.4	0.0	0.0
CD200111	77.5	75.5	8.4	6.6	0.0	0.4
CD113	87.5	72.5	5.7	5.1	0.6	0.0
CD200117	83.0	65.5	6.6	12.2	0.6	1.3
CD105	76.5	68.5	7.8	5.1	0.4	0.1
CD200126	80.5	69.5	15.5	33.0	0.3	1.0
CDFAPA 2001129	97.0	88.0	25.0	33.5	0.1	0.3

MMS= Modified Murashige and Skoog medium
MS= Murashige and Skoog medium

The explanation given by some authors for the great effect of the genotype is the variance in the endogenous hormone levels. The application of exogenous growth regulators combined with the production of endogenous hormones under genetic and environmental control could influence the hormonal balance, favoring organogenesis or embryogenesis, thus, allowing specific genotypes responses in determined media (Jiménez and Bangerth 2001, Li et al. 2003). In relation to wheat, Tamás et al. (2004) showed that the endogenous hormone balance influences the regeneration of green spots as well as the appearance of plantlets. The authors detected an interaction between genotype, type of explant, composition of the culture medium and cultivation conditions determining the genotype regeneration capacity. On the other hand, Li et al. (2003) observed that the effect of the genotype cannot be attenuated by simply manipulating the medium composition or even by using another experimental parameter.

The maximization of *in vitro* culture conditions, with the development of efficient protocols of tissue culture and the choice of responsive wheat genotypes, is important for plant improvement programs by shortening the time needed to breed new varieties, by using haploids and for genetic transformation. The results presented here demonstrated the different capacity of callus induction and plant regeneration of the evaluated genotypes, which makes it possible to choose the best genotypes to achieve such characteristics, by optimizing the growing process of immature embryo culture and the plant regeneration process.

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Indução de calos e regeneração de plantas em genótipos elite de trigo brasileiros

RESUMO – *Os primeiros passos para a produção de plantas transgênicas são a distinção de genótipos responsivos à cultura de tecidos e o desenvolvimento de um sistema eficiente de regeneração. Nove genótipos de trigo (*Triticum aestivum *L.) de cultivares melhoradas pela Coodetec foram cultivados in vitro com o objetivo de avaliar a competência para a formação de calos e embriogênese somática. Os explantes (embriões zigóticos imaturos) foram testados em dois meios distintos, MS (Murashige e Skoog 1962) e MS modificado - MMS (Zhou et al. 1995) com dosagens decrescentes de reguladores de hormônios, num sistema composto de três fases distintas de cultivo: indução de calos, manutenção da cultura e regeneração de plantas. Após a indução, os calos foram avaliados a cada 21 dias quanto suas respostas à embriogênese somática. Os resultados demonstram uma resposta diferenciada dos genótipos dependendo do meio de cultura usado. O genótipo CD104 apresentou os melhores resultados de resposta embriogênica nos dois meios de cultura. Em MMS, esse genótipo obteve valores de 100%, 99.5% e 1.1%, para indução de calos, regeneração de plantas e relação entre plântulas regeneradas e embriões resgatados, respectivamente. Além do CD104, os genótipos CD200126 e CDFAPA 2001129 foram os mais responsivos no meio MS, com valores de 37.5%, 33.5% e 33% respectivamente para a capacidade de regeneração, tornando-os candidatos potenciais para transformação genética em programas de melhoramento de planta.*

Palavras-Chave: Trigo, *Triticum aestivum*, indução de calos, regeneração de plantas, meios de cultura, embriogênese.

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