BRIEF COMMUNICATION

Callus induction and plant regeneration from immature embryos of *Brachypodium distachyon* with different chromosome numbers

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Abstract

The paper reports the *in vitro* cultivation of two commercial lines and 23 wild populations (with 10, 20 and 30 chromosomes) of *Brachypodium distachyon*. Callus induction was assayed on Murashige and Skoog medium containing 1 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) with 30 g dm^{-3} of sucrose (MSs) or maltose (MSm). No significant differences were seen between the two media with respect to callus induction. Calli were transferred to MSm medium without 2,4-D but containing 0.1 mg dm^{-3} of 6-benzylaminopurine for plant regeneration. The plant regeneration response was very variable depending on the original induction medium, although no overall preference for one or the other medium was seen. The three main culture stages (callus induction, plant regeneration, and green plantlets formation) are probably differently controlled in the plants with different chromosome numbers. This supports the idea that the three cytotypes of *Brachypodium* cultured actually belong to different species.

Additional key words: *in vitro* culture, auxin, cytokinin, maltose, saccharose.

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Brachypodium distachyon (L.) Beauv. is phylogenetically related to cereals of great economic importance, including wheat and barley (Catalán and Olmstead 2000), and its use as a model for the genomic analysis of the grasses of temperate climates has been proposed (Draper *et al*. 2001, Opanowicz *et al*. 2008). The species is diploid (2n=10) with a small genome of about 160 Mpb and is autogamous. In addition to the 10-chromosome cytotype, forms with 2n=20 and 2n=30 have also been described; these have been proposed autotetraploids and allohexaploids respectively (Robertson 1981, Draper *et al*. 2001). *Brachypodium distachyon* has been used to protect rural soils from erosion, especially in sloping areas where runoff, the wind and other agents cause the loss of soil, rendering the land unsuitable for agriculture. A number of varieties of *B. distachyon* useful for protecting olive groves have been marketed in Spain (Soler *et al*. 2004).

For a species to be deemed a biotechnological model of a botanical group, the culture of its tissue and the eventual regeneration of plants from these cultures need to be relatively easy. Immature embryos, mature embryos or leaf base segments are the most common starting material for establishing embryogenic calli of cereals and grasses and subsequent plant regeneration (Bohorova 2001, González *et al*. 2001, Dahleen and Bregitzer 2002, Eudes *et al*. 2003, Dabul *et al*. 2009, Li *et al*. 2009, Ozbay and Özgen 2010). Currently, procedures are available that permit the *in vitro* culture of genetically modified *Brachypodium*. However, few genotypes have been used (Bablak *et al*. 1995, Draper *et al*. 2001, Christiansen *et al*. 2005, Vogel *et al*. 2006, Vain *et al*. 2008, Vogel and Hill 2008).

In the present work, plants belonging to wild populations of *Brachypodium distachyon* plus two marketed lines of the taxon, with different chromosome numbers (10, 20 and 30 chromosomes) and two different culture media were assayed, in order to determine the

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Abbreviations: BA - 6-benzylaminopurine; 2,4-D - 2,4-dichlorophenoxyacetic acid; MSm - Murashige and Skoog medium with maltose; MSs - MS medium with saccharose.

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influence of genotype, chromosome number and culture medium on the induction of calli produced from immature embryos of plants and the capacity to regenerate plants from the calli. The results could be used in future genetic transformation programs.

The variability of the *in vitro* culture response was studied in 23 populations of *Brachypodium distachyon* (L.) Beauv. collected from wild populations of the Iberian Peninsula. The collection is maintained by the authors of the Departamento de Medio Ambiente, I.N.I.A. The chromosome number determination, seed germination, root treatment and mitotic chromosome preparations were performed as described by Cuadrado and Jouve (2007). Chromosomes were stained with DAPI, and examined under a *Zeiss Axiophot* microscope (*Carl Zeiss*, Oberkochen, Germany). The wild populations had 10, 20 or 30 chromosomes. The commercial cultivars Zulema and Ibros had 10 and 30 chromosomes, respectively.

From 60 to 160 immature embryos (0.5 - 0.7 mm long) coming from 15 plants per population or cultivar were distributed in three replicates and were aseptically removed from the caryopses and placed scutellum-side-up in Petri dishes containing one of two callus induction media. Both these media were based on Murashige and Skoog (1962) medium with 1 mg dm^{-3} 2,4-dichloro-phenoxyacetic acid (2,4-D). Medium MSs medium contained 30 g dm^{-3} sucrose, while MSm medium contained 30 g dm^{-3} maltose. The immature embryos were incubated in the dark at 26 ± 1 °C for 4 weeks and then the number and type of calli were recorded.

The calli obtained from the immature embryos on the different induction media were transferred to a regeneration medium (RMSm), *i.e*., MSm lacking 2,4-D but incorporating 0.1 mg dm^{-3} 6-benzylaminopurine (BA). All calli were placed in a growth incubator under fluorescent lamps for 4 weeks (temperature of 22 ± 1 °C, 14-h photoperiod, irradiance of 75 μmol m^{-2} s^{-1}) and the number of green or albino shoots obtained per callus was recorded. Data were analyses using the *StatGraphics Plus* (*7.0* version) program. The mean values for each of the variables recorded during callus induction and plant regeneration were compared using the Student *t*-test.

The calli began to develop during the second week of culture, and were classified into two types according to their appearance under a stereo microscope: *1*) soft calli (watery and translucent or light yellow) or *2*) compact embryogenic calli of yellowish colour. The number of total calli/number of embryos plated (TcE) and the number of compact embryogenic calli/total number of calli (Ec/Tc) were used to compare induction capacity between different media and genotypes.

After four weeks on the induction media, the calli were transferred to regeneration medium for a further four weeks. The number of green and albino plants obtained was recorded with respect to the induction medium on which the calli were originally raised and the type of callus used. Plants were regenerated both from compact embryogenic and soft calli, although the latter type produced fewer plants. The following variables were calculated from the data obtained: number of green plants obtained from compact embryogenic calli/total number of compact embryogenic calli (Egp/Ec), the number of green plants obtained from soft calli/the total number of soft calli (Sgp/Sc), the total number of green plants/total number of calli (Gp/Tc), the total number of albino plants/total number of calli (Ap/Tc), and the total number of green plants/total number of immature embryos (Gp/E).

The culture medium had no significant influence on the callus induction response (Table 1). Data for both induction media were therefore pooled for the following analyses. Pearson product-moment correlation coefficients were calculated to detect possible relation-ships between the studied induction and regeneration response variables. Significant, positive correlations were found between Gp/Tc and Egp/Ec, Gp/Tc and Sgp, Gp/Tc and Gp/E, and Gp/E and Egp/Ec. One-way *ANOVA* was used to detect differences between plants with different numbers of chromosomes in terms of the callus induction and regeneration response. To normalize the distribution of the variables, the data were first arcsine $\sqrt{x}/100$ transformed. Comparison of the mean values was undertaken using Fisher's least significant difference (LSD) test. The significant differences were detected with

Table 1. Influence of induction medium used (MSs and MSm) on the callus induction and plant regeneration responses of the different populations and lines of *Brachypodium* classified by chromosome number. Means ± SD of three replicates per population and medium**.**

Material	Medium	TcE	Ec/Tc	Egp/Ec	Gp/Tc	Gp/E	Sgp/Sc	Ap/Tc
All plants	MSs	0.72±0.30	0.38±0.27	1.57±1.88	0.86±0.99	0.60±0.51	0.39±0.45	0.04±0.10
	MSm	0.61±0.32	0.36±0.26	2.44±2.64	1.15±1.35	0.69±0.88	0.25±0.30	0.06±0.15
2n=10	MSs	0.83±0.16	0.20±0.15	0.89±1.00	0.64±0.58	0.49±0.49	0.51±0.60	0.01±0.02
	MSm	0.61±0.31	0.15±0.11	1.42±2.46	0.49±0.55	0.38±0.50	0.30±0.23	0.02±0.06
2n=20	MSs	0.56±0.42	0.82±0.24	0.45±0.37	0.35±0.22	0.22±0.22	0.08±0.14	0.22±0.18
	MSm	0.41±0.52	0.86±0.19	0.44±0.08	0.36±0.02	0.21±0.19	0.00±0.00	0.05±0.07
2n=30	MSs	0.69±0.34	0.41±0.19	2.31±2.28	1.16±1.27	0.78±0.53	0.38±0.35	0.03±0.06
	MSm	0.66±0.28	0.41±0.20	3.21±2.69	1.68±1.58	0.95±1.05	0.26±0.34	0.08±0.19

respect to the variables TcE, Ec/Tc and Ap/Tc (Table 2). The induction of calli by the *in vitro* culture of immature embryos is influenced by a number of factors, among which the most important are the composition of the induction medium and the genotype. Several studies on the effects of different components of the culture media, usually involving variable carbon source (*e.g*. sucrose or maltose), have been undertaken. However, the results obtained have been contradictory and dependent on the species, genotype and *in vitro* culture phase in question (González and Jouve 2000, González *et al*. 2001, Mendoza and Kaeppler 2002, Dahleen and Bregitzer 2002, Eudes *et al*. 2003).

Table 2. Results of the Fisher's LSD test for the variables analysed. Populations with the same letter showed no significant differences at $P = 0.05$.

Plants	TcE	Ec/Tc	Egp/Ec	Gp/Tc	Gp/E	Sgp/Sc	Ap/Tc
2n=10	a	a	a	a	a	a	a
2n=20	b	b	a	a	a	a	b
2n=30	ab	c	a	a	a	a	ab

Procedures have been also developed for the regeneration of plants via the *in vitro* culture of both mature (Bablak *et al*. 1995) and immature embryos (Draper *et al*. 2001, Vogel and Hill 2008) of *Brachypodium*. However until now, the responses of different *B. distachyon* genotypes had not been compared. In the present work, the callus induction response induced by both media was similar (Table 1). No significant differences were seen in the induction response variables either for plants of different population/line or chromosome number.

The plant regeneration results obtained in the present study appear to indicate that the most important factor, in terms of the regeneration response, is the interaction between the genotype and callus induction medium rather than the type of medium used *per se*. It is very difficult to define a single medium in which all genotypes behave optimally.

The results mentioned above suggest that at least three different stages, probably controlled by different genetic systems, must be passed through in the regeneration of *Brachypodium* plants: callus induction, plant regeneration, and green plant production (Chowdhury *et al*. 1991, Bohorova *et al*. 2001).

The materials used in the present work included plants with three different chromosome constitutions. These three cytotypes of *Brachypodium* are currently considered to represent three different species (Hasterok *et al*. 2004, Idziak and Hasterok 2008, Filiz *et al*. 2009). Two of these are diploids with 2n=10 and 2n=20; the third is allotetraploid (2n=4x=30 chromosomes), a product of hybridisation and chromosome doubling. One-way *ANOVA* showed significant differences between the plant materials with different chromosome constitutions in terms of TcE, Ec/Tc and Ap/Tc (Table 2). It therefore follows that chromosome constitution affects not only the capacity to induce calli, but also the production of compact embryogenic calli and the proportion of albino plants regenerated from immature embryos.

The diploid plants with 2n=10 showed the greatest callus-forming ability, followed by those with 2n=30, and finally those with 2n=20. The representatives of these three levels of ploidy showed significant differences with respect to the proportion of compact embryogenic calli produced, the 2n=20 plants being the best. The 2n=10 plants generated the fewest.

Finally, although the proportion of albinism was low in all cases, the plants with 20 or 30 chromosomes generated the higher proportions of albino plants than plants with 10 chromosomes. These results reflect different, chromosome-number-dependent *in vitro* culture behaviours. The present results also add to the number of known differences between the plants belonging to the three different cytotypes. Plants of the commercial cultivar Zulema (2n=10) and the wild populations Bd115 (2n= 20) and Bd341 (2n=30) showed the best fitness for the *in vitro* culture of immature embryos. These genotypes could be considered good basic material for use in future genetic transformation programs.

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