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Flow cytometry as a tool for analyses of soya bean seed vigour

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Abstract

Vigour tests of soya bean seeds are important in seed production; however, many of them have a number of steps and are time-consuming. The analysis of the cell cycle / endoreduplication by flow cytometry can be an alternative to supplement seed quality analyses. Therefore, the aim of this study was to evaluate the potential of flow cytometry to assess soya bean seed vigour compared with other commonly used vigour tests. Four lots of soya bean seeds were subjected to a standard germination test, accelerated ageing test, electrical conductivity test, seedling emergence in sand, and computerised image analysis of seedlings (SVIS®) Dry seeds and those hydrated for 24 hours were evaluated by flow cytometry to determine cell cycle activity and endoreduplication intensity in different seed parts (embryo axis and cotyledons). Seed lots determined by the conventional tests as having high quality, possessed a greater proportion of cells with the highest DNA contents (4C in the embryo axis and 8C in the cotyledons) than those of lower quality. It is suggested that in these lots activation of mitotic cycle / endoreduplication during imbibition was faster and therefore they expressed higher vigour. In conclusion, flow cytometric analysis of seeds has the potential for a fast and reliable evaluation of soya bean seed vigour.

Keywords: cell cycle, DNA content, endoreduplication, germination, Fabaceae, *Glycine max*

Introduction

In the production of soya bean seeds, testing procedures are commonly performed to assure high quality lots to farmers. Most of the tests are based on counting seedlings after the completion of germination (ISTA, 1993, 1995). Many vigour tests have a number of steps and are time-consuming and some are not fully reliable. Therefore, there is a need to develop faster and more accurate methods to assess seed quality. One of the processes

that take place during Phase II of germination is DNA synthesis, which is indicative of activation of the cell cycle (Nonogaki *et al*., 2010). A fast and accurate method to estimate nuclear DNA content is flow cytometry and therefore it can be used to determine cell cycle activity, which corresponds with the advancement of germination (Bino *et al*., 1992, 1995; Pawlowski *et al*., 2004; Gendreau *et al*., 2008; Rewers *et al*., 2009; Sliwinska, 2009; Forti *et al*., 2015).

The first phase of the cell cycle, G_1, is the first phase of cell growth, when a nucleus contains 2C DNA (C = DNA content of a holoploid genome with chromosome number *n*). During the following phase, S, DNA replication occurs, which results in a doubling of DNA content (2C to 4C). Afterwards, in the G_2 phase, a second growth period occurs, during which the nucleus retains a 4C DNA content; finally there is division into two daughter nuclei during mitosis (M). Cells that leave the cell cycle, usually from the G_1 phase, enter the quiescent G_0 state (Bewley and Black, 1994; Bino *et al*., 1992; Vázquez-Ramos and de la Paz Sánchez, 2003). Some cells, however, for example those of the endosperm or cotyledons, undergo endoreduplication. In this process nuclei go through repeated rounds of DNA replication that are not followed by mitosis, resulting in cells possessing 4C, 8C, 16C, 32C, etc., DNA (Bino *et al*., 1993; Breuer *et al*., 2014).

Previous studies revealed that an increase in DNA replication activity in seed cells marks the advancement of germination of sugarbeet (Sliwinska and Jendrzejczak, 2002), coffee (Da Silva *et al*., 2008), barley (Gendreau *et al*., 2008) and soya bean (Forti *et al*., 2015) seeds. The increase in proportion of cells with 4C DNA content is an indicator of the transition of the seed to Phase II of germination (Bewley and Black, 1994) and therefore can be considered as an appropriate marker for seed quality (for review see Sliwinska, 2009). Therefore, the 4C / 2C ratio has been recommended as a marker of seed quality. However, for the seeds / seed parts where endoreduplication occurs, the ($\sum >$ 2C) / 2C ratio, which includes endopolyploid cells, should be used instead (Rewers and Sliwinska, 2012).

Vigour tests have been proposed to identify differences associated with seed lot performance during storage or after sowing, in order to check the seed lot potential for high field establishment over a wide range of environmental conditions (Marcos Filho *et al*., 2009). Several tests have been recommended for soya bean seed vigour evaluation, including accelerated ageing, tetrazolium, electrical conductivity, seedling growth, visual classification of seedling morphology (Vieira *et al*., 2003) or the computerised image analysis of seedlings by the Seed Vigor Imaging System (SVIS®) (Wendt *et al*., 2014).

The aim of the present study was to evaluate the potential of flow cytometric analyses to assess soya bean seed vigour as compared with the vigour tests commonly used for this species.

Materials and methods

Four soya bean seed lots of cultivar TMG115-RR were used for germination and seed vigour tests according to the methods described below.

Germination (G): Five replicates of 40 seeds from each seed lot were sown in a paper towel moistened with water equivalent to 2.5 times seed weight and incubated at 25°C in a germination chamber. Counting of germinated seeds was made on the eighth day after the beginning of imbibition (Brasil, 2009).

Accelerated ageing (AA): Five replicates of 40 seeds for each seed lot were distributed in transparent plastic boxes (100 × 100 × 40 mm), on wire mesh containing 40 mL water. These boxes were kept in an ageing chamber for 41°C for 48 hours. After that, the seeds were set to germinate and the seedlings were evaluated as described for the germination test.

Electrical conductivity (EC): Five replicates of 25 seeds for each seed lot were weighed and soaked in 75 mL distilled water for 24 hours in a controlled temperature chamber at 25°C (Custódio and Marcos Filho, 1997). The electrical conductivity of the solutions was then evaluated and the results expressed in $\mu S\ cm^{-1}\ g^{-1}$.

Seedling emergence in sand (SES): Four replicates of 100 seeds for each seed lot were sown in trays containing sand as substrate. Fourteen days after sowing, the percentage of normal seedlings was determined.

Computerised image analysis of seedlings (SVIS®): Four replicates of 50 seeds of each seed lot were distributed in the upper third of a paper towel sheet and maintained at 25°C, for three days. The seedlings were transferred to a sheet of Black Bristol paper and placed in a scanner (Scanjet 2004), fastened upside-down inside an aluminum box with dimension of 600 × 500 × 120 mm and analysed by the software Protosmart®, with resolution of 98 dpi. The seedling images were then analysed by the Seed Vigor Image Seedlings (*SVIS®*) software to obtaining the mean values of vigour index (VI), growth (GI) and uniformity (UI) indices and the length of seedlings (SL) for every seed lot (Marcos Filho *et al*., 2009).

Flow cytometry: The embryos of dried and hydrated (24 hours at 25°C) seeds were dissected into embryonic axis and cotyledons, and analysed by flow cytometry. Samples of individual seed parts were prepared as previously described (Rewers *et al*., 2009), using nuclear isolation buffer (0.1 M Tris-Cl, 2.5 mM $MgCl_2 \cdot 6H_20$, 85 mm NaCl, 0.1% v/v Triton X-100, pH 7.0), supplemented with 4', 6-diamidino-2-phenylindole (DAPI; 2 mg mL^{-1}) and 2% (w/v) polyvinylpyrrolidone-10 (PVP-10). Analyses were performed on 10 biological replicates, using logarithmic amplification of the signal. For each sample, at least 7000 nuclei were analysed using a Partec CCA (Partec GmbH, Münster, Germany) flow cytometer, equipped with an HBO lamp. Histograms were evaluated using the DPAC v. 2.2 programme (Partec GmbH, Münster, Germany). The proportion of nuclei with different DNA contents and the 4C + 8C / 2C ratio were calculated.

Statistical analysis: The results were analysed statistically using a one-way analysis of variance and the Tukey's test ($P = 0.05$). The percentage data from the germination tests were subjected to ANOVA after angular transformation; actual percentages are presented in the tables.

Results

Germination and vigour tests revealed differences in seed quality between the seed lots (table 1). Lots 1 and 2 demonstrated higher germination in the standard test (G) and after accelerated ageing (AA) than lots 3 and 4. They also exhibited higher seedling emergence in sand (SES). However, the electrical conductivity test (EC) revealed that seeds of lot 1 had lower membrane integrity (high conductivity), similar to lots 3 and 4. The evaluation by SVIS® revealed that lot 4 possessed a higher vigour index (VI) and growth index (GI) than lot 3, while lots 1 and 2 were similar to both other lots for these parameters. The uniformity index (UI) and the seedling length (SL) did not exhibit differences between seed lots. Thus, lots 1 and 2 can be considered as possessing high vigour (although lot 1 was slightly poorer because of lower membrane integrity), and lots 3 and 4 as possessing lower vigour, with lot 4 being slightly better.

Table 1. Germination in a standard test (G) and after accelerated ageing (AA), seedling emergence in sand (SES), electrical conductivity (EC), and parameters analysed by the Seed Vigor Imaging System (SVIS®), vigour index (VI), growth index (GI), uniformity index (UI) and seedling length (SL), of four soya bean seed lots. CV - coefficient of variation.

Seed lot	G (%)	AA (%)	SES (%)	EC (μS cm^{-1} g^{-1})
1	95 a*	99 a	99 a	41.04 a
2	97 a	98 a	97 ab	29.49 b
3	90 b	93 b	96 b	33.32 ab
4	90 b	93 b	94 b	35.77 ab
CV (%)	3.21	2.02	2.30	13.35
Seed lot	SVIS			
	VI	GI	UI	SL (mm)
1	683.0 ab	599.5 ab	880.0^{NS}	59^{NS}
2	654.5 ab	562.0 ab	868.5	56
3	642.0 b	542.5 b	877.0	52
4	762.5 a	688.8 a	874.3	64
CV (%)	8.94	13.53	3.11	16.03

*values for a particular parameter (in columns) followed by the same letter are not significantly different at $P = 0.05$ (Tukey's test)
^{NS} no significant difference

Flow cytometric analysis revealed that in the embryo axis, only 2C and 4C nuclei were present, while in the cotyledons, endopolyploid 8C nuclei also occurred (table 2). In both organs, the majority of the nuclei possessed 2C DNA; in the embryo axis they comprised 95% of total and in the cotyledons about 70%. In dry seeds the proportions of nuclei with 2C and 4C DNA as well as the 4C + 8C / 2C ratio were similar for all lots; however, in the cotyledons of lots 1 and 2 there were more 8C nuclei (about 7%) than in lots 3 and 4 (about 5%). Upon hydration the proportions of nuclei with higher DNA content increased,

but to different extents in different lots, which allowed distinction between seed lots in regard to advancement of germination after 24 hours from the start of imbibition. In the embryo axis of lots 1 and 2, a higher proportion of 4C nuclei occurred than in lots 3 and 4, which is indicative of higher cell cycle activity in the lots that were considered to be of high-vigour. In the cotyledons a similar tendency was observed regarding nuclei with the highest DNA content (8C); their proportion was higher in high-vigour lots. Consequently, the 4C + 8C / 2C ratio was also higher in both organs of lots 1 and 2.

Table 2. Percentage of nuclei with different DNA contents and the 4C + 8C / 2C ratio in the axis (A) and cotyledons (C) of dry (D) and 24-hour-hydrated seeds at 25°C (H) of four soya bean seed lots. CV - coefficient of variation.

Seed lot		Percentage of nuclei with DNA content						4C + 8C / 2C ratio	
		2C		4C		8C			
		A	C	A	C	A	C	A	C
1	D	95.1^{NS}	68.9^{NS}	4.9^{NS}	24.2^{NS}	0	6.9 a	0.051^{NS}	0.451^{NS}
2		95.5	67.6	4.5	25.0	0	7.4 a	0.047	0.479
3		95.1	69.8	4.9	25.7	0	4.5 b	0.051	0.432
4		95.0	71.0	5.0	23.9	0	5.2 b	0.053	0.409
CV(%)		2.25	4.34	17.23	9.20	-	42.3	16.7	18.4
1	H	90.8 c*	61.6 b	9.2 a	27.8^{NS}	0	11.1 a	0.101 a	0.636 a
2		91.1 bc	60.6 b	8.9 a	27.1	0	12.4 a	0.098 a	0.651 a
3		92.8 ab	66.9 a	7.2 b	26.1	0	7.0 b	0.078 b	0.495 b
4		93.2 a	66.4 a	6.8 b	25.6	0	7.9 b	0.073 b	0.505 b
CV(%)		1.77	6.76	20.30	10.85	-	36.37	22.57	20.46

*values for a particular germination stage (in columns) followed by the same letter are not significantly different at $P = 0.05$ (Tukey's test)
^{NS} no significant difference

Discussion

Flow cytometry has been used for the estimation of seed quality and advancement of germination since the 1990s, and initially the radicle or radicle tip was considered to be the most suitable material for analysis (Bino *et al*., 1992, 1995; de Castro *et al*., 1995; Sliwinska and Jendrzejczak, 2002; Pawlowski *et al*., 2004; Faria *et al*., 2005; Gendreau *et al*., 2008). However, current research reveals that during germination the most intensive DNA synthesis occurs in the radicle / hypocotyl transition zone and in the hypocotyl, and these embryos regions should be included when conducting molecular research on germination (Sliwinska *et al.*, 2009; Rewers and Sliwinska, 2014). Therefore, the whole embryonic axis rather than just the radicle was used in this study for flow cytometric analysis. Since cotyledons in the Fabaceae family are the major storage organs in a mature seed, and cell cycle / endoreduplication intensity can mark germination stages (Rewers and Sliwinska, 2014), they were also included in the analyses.

Different seed vigour tests applied here yielded comparable results and allowed classification of seed lots into two groups: 1 and 2 were considered as high-vigour and 3 and 4 as lower-vigour. However, such standard tests take a long time to be completed (up to 8-14 days) and sometimes they are not sensitive enough to detect vigour differences between seed lots. Therefore, there is a need to develop faster and more accurate methods; flow cytometry is a useful alternative.

The embryo of quiescent mature seeds predominantly contains nuclei possessing a 2C DNA content, indicating the arrest of the cell cycle at the G_0 / G_1 stage (Sliwinska, 2009). During germination the cell cycle resumes and DNA in some cells replicates, reaching 4C (Vázquez-Ramos and de la Paz Sánchez, 2003). This is also evident for soya bean embryo axes in all seed lots analysed here. When seeds were subsequently imbibed in water for 24 hours, the proportion of 4C cells (at the G_2 phase of the cell cycle) increased, in preparation for cell division, which usually occurs after germination is completed (Nonogaki *et al*., 2010). However, as reported before (Rewers and Sliwinska, 2014), later during germination, some of the 4C axis cells in soya bean seeds leave the mitotic cycle and undergo endoreduplication. This coincides with cell enlargement and therefore is one of the mechanisms participating in axis elongation and completion of germination. The present results confirmed those obtained for sugar-beet seeds, which revealed that in high-vigour lots the increase in the proportion of 4C nuclei during germination was much faster than in low-vigour lots (Sliwinska and Pedersen, 1999). High-vigour seeds need a shorter period for DNA repair than low-quality seeds (Osborne, 1977).

The pattern of DNA synthesis was different in the cotyledons because of endoreduplication, which is typical for this organ in the Fabaceae family (Rewers and Sliwinska, 2012, 2014). Nevertheless, also in the cotyledons, an increase in the proportion of nuclei with the highest DNA content (8C) was evident. This increase in the cotyledons, as in the axis, was greater in lots 1 and 2 then in lots 3 and 4. Since endopolyploid cells usually have an increased volume, the present results suggest that in high-vigour seeds cotyledons can more efficiently synthesise the compounds necessary for germination and early seedling growth.

Similar to the proportion of the nuclei with different DNA contents, the $4C + 8C / 2C$ ratio, which gives an indication of changes of the proportion of 2C nuclei in relation to nuclei possessing higher DNA contents (activation of cell cycle and/or endoreduplication), was suitable to distinguish between low- and high-vigour lots. In both seed parts, the axis and cotyledons, this ratio was higher in lots of higher quality. To the best of our knowledge, the relation between endoreduplication intensity and seed vigour has not been reported previously.

In conclusion, flow cytometry performed on soya bean seeds as early as after 24 hours from the start of imbibition provides information on seed vigour, and the data obtained are in agreement with other tests that take a much longer time, such as the standard germination test (eight days), accelerated aging test (48 hours of treatment and eight days of germination), and some of the parameters obtained using the Seed Vigor Imaging System (three days). Thus, flow cytometry has potential to be used for soya bean seed lot vigour screening.

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