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Pollen viability of sugarcane after storage

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ABSTRACT

Knowledge about conservation of pollen viability in sugarcane allows estimation of the preservation time, germinative power, vigor and genetic integrity of the pollen, and may facilitate crosses between asynchronous flowering parents. This work evaluated the viability of pollen in the upper and lower third of the panicles in ten genotypes and the viability during the storage time in four genotypes. The collected pollen grains were stored in a freezer at -18°C and the viability was evaluated by means of a 2% Lugol solution. The evaluations were performed at ten-day intervals, during a fifty-day period. Significant differences were observed for the genotype x panicle interaction of four of the ten evaluated genotypes, where genotypes G2, RB813804 and RB931011 presented higher viability in the lower third, while genotype RB863129 presented greater viability in the upper third of the inflorescence. Significant differences were also observed for the genotype x storage days interaction for the four genotypes. It was verified that the genotypes present a differentiated behavior in the loss of viability and that the accessions RB813804, RB931011 and RB863129 remained viable in the final evaluation.

INTRODUCTION

The exploitation of genetic variability, available in the Active Banks of Germplasm (ABG) of sugarcane, is essential for the selection of generics in segregating populations, obtained through artificial crosses. The product of such crosses is like caricatures, from which they are produced in the seedlings that enter the cycles of selection, multiplication, experimentation and validity, until a release of the new variety for commercial production (Simões Neto et al., 2005). According to Cesnik and Miocque (2004), the main methodologies for carrying out hybridizations of *Saccharum* in Brazil are the multiple crossings (MP) or polycrossing (PL), special multiple crossings (MPE), self-fertilizations and mainly biparental crosses (BP); which stand out by allowing a maximum firm of heterosis.

However, in the genetic improvement of sugarcane, cross-breeding, mainly of BP, has as a limiting factor, the lack of flowering synchronism between the parents. The main methodologies to overcome this problem are: Stepwise planting of accesses, use of darkroom with photoperiod control and application of inducers or flowering inhibitors (Araldi et al., 2010).

However, these procedures and their costs are high and/or they have low efficiency (Amaral et al., 2012). Another methodology used to make hybridizations between genotypes that do not show flower synchrony and between plants that are in androsthetic, dichogamic or self-incompatible is the conservation of pollen (Song and Tachibana, 2007; Damasceno Junior et al., 2008). This methodology is also important because it allows the exchange of polylines between research centers (Hanna, 1994).

Several techniques are used for the conservation of pollen, among them, only cryopreservation (storage in liquid nitrogen at -196ºC) can guarantee the storage of germplasm in the long term (Kaviani, 2011). Pollen storage at low temperatures (-18 and -72ºC) presents low

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Table 1. Identification of sugarcane genotypes, evaluated for pollen viability, with their parents and origin.

Genotypes	Parents		Origin
	Female	Male	
G2 *	SP80-1816	***	UFRPE / RIDESA
G3 *	ROC3	RB83100	UFRPE / RIDESA
G9 *	R397	***	UFRPE / RIDESA
G10 (X) *	RB943365	-	UFRPE / RIDESA
RB002504**	SP80-1816	***	UFRPE / RIDESA
RB867515 **	RB72454	***	UFV / RIDESA
RB872552 **	RB754665	RB773720	UFRPE / RIDESA
RB813804 **	CP48-124	***	UFRPE / RIDESA
RB863129 **	RB763411	***	UFRPE / RIDESA
RB931011 **	RB83160	RB72454	UFAL / RIDESA

Clones in experimentation; Commercial ** Range; *** Genitor unknown; (X) selfing.

implementation costs and is efficient for pollen conservation of sugarcane (Amaral et al., 2012; Melloni, 2012).

It is considered as technical conservation of resources; develop with the resources for viability. Such analyses can be done by direct (*in vitro*) and/or indirect (cytological labeling) methods. Among these methods, a cytological labeling has lower and faster cost (Aslantas and Pirlak, 2000). Among the cytological markers most used by the indirect method, the following stand out: Alexander dye, acetic carmine, propionic carmine, acetic orcein, lactophenol blue and lugol solution. However, according to Amaral et al. (2012), lugol solution is the most used method to test for the pollen viability of sugarcane.

Also, according to Amaral et al. (2012), pollen viability analyses are carried out on pollen grains collected from the upper third of the inflorescence, as well as the ones onwards. However, up till date, the potential of the basal third of the inflorescence for collecting and preserving the sugarcane stick is not known. Likewise, there is little information in the literature regarding the viability of pollen and the time at which pollen from the main genitors explored in hybridizations remains viable when subjected to low temperature conservation.

The objective of this work was to study sugarcane genotypes that show asynchronous flowering and the viability of their pollens obtained from the upper and lower third of the panicle; as well as an endless pollen viability of storage time, the period in which they remain fertile for hybridizations.

MATERIALS AND METHODS

Ten sugarcane genotypes were used, four promising clones and six commercial varieties (Table 1); chosen because they present agro-industrial characteristics important for exploitation in sugarcane genetic improvement programs, such as high agricultural and high productivity sucrose content (Costa, 2012).

Initially, ten genotypes were evaluated for the pollen viability of the upper third and lower third of the panicle. Subsequently, genotypes RB872552, RB813804, RB863129 and RB931011 were evaluated for pollen fertility over their shelf life.

In order to collect the pollen grains, the genotypes were planted at the Germoplasma Active Bank (GAB) of the Flowering and Crossing Station of the Sugar Cane of Devaneio (EFCCD) belonging to the Experimental Sugarcane Station of Carpina (EECAC) of the Federal Rural University of Pernambuco (UFRPE), member of the Inter-University Network for Development of Sugar and Alcohol Sector (RIDESA).

The EFCCD is located in the Municipality of Amaraji, Zona da Mata Sul of the State of Pernambuco, latitude 08° 19" 8' S, longitude 35° 24" 893' W and altitude 514 m. The average annual precipitation is 2600 mm, with minimum and maximum temperatures of 18.92 and 28.15°C, respectively. The local climate is tropical with dry season. This environment is considered conducive to the flowering of sugarcane, and it is possible to carry out hybridizations (Silva et al., 2013).

The planting of each parent was carried out in two 5.0 m furrows, spaced 1.0 m between furrows and 0.5 m between clumps. According to Pastina et al. (2012), in order to facilitate the handling of the stalks in the act of collecting the panicle and performing the crosses, as well as to promote the conservation of the inflorescences and the maintenance of the pollen viability after the cut of the stalks, it is better to use traditional acid solution, also described by Amaral et al. (2012). However, in order to achieve this step of the crossing protocol, the procedure is replaced by performing ten alp ores in each genotype.

Pollen viability analyses were performed in the EFCCD

Table 2. Analysis of pollen viability of the upper and lower third of the panicle of sugarcane genotypes.

Sources of variation	Degrees of freedom	Sum of squares	Mean square
Genotypes (G)	9	1.1497	0.1277 **
Section panicle (S)	1	0.1148	0.1148 **
Section genotype x (S x L)	9	0.7573	0.0841 **
Residue	60	0.7029	0.0117 **
Total fixed	79	2.7246	

Coefficient of variation (%) = 11.71
**Significant at 1% probability level for the F test.

laboratory and the pollen collection procedures followed the methodology proposed by Amaral et al. (2012), with the following modification: panicles were collected showing the upper third in a thesis and others showing the lower third in a thesis. After collection, the pollen grains were packed in 100 mL glass stopper flasks, which were kept open in a glass desiccator with 1 kg of silica gel blue at 4°C for 1 h period for dehydration. Afterwards the pots were closed and stored in the freezer at -18°C. Finally, samples of pollen grains were obtained from six mature anthers, that were deposited and opened on a histological slide.

The pollen viability was analyzed by means of cytological labeling with 2% lugol solution (1 g of iodine, 2 g of potassium iodide and 100 ml of distilled water) according to Amaral et al. (2012). According to these authors, in this method pollen is considered to be viable when it presents brownish tones, this occurs due to the presence of starch reserve and the integrity of membranes, while uncolored, pale yellow and/or translucent pollen grains are considered infeasible or malformed.

The counting of the stained and uncoloured pollen grains allowed for estimating the pollen viability, expressed as a percentage (%VP), through the formula:

$$VP\ (\%) = \frac{\text{Number of pollen grains stained x 100}}{\text{Number of total pollen grains}}$$

The data obtained in percentage were transformed to sine wave √x (%) for posterior analysis of variance. According to (Ferreira, 2000), such transformation must be performed when the data come from binomial distribution and extrapolate the amplitude from 30 to 70%.

The experimental design adopted for the two experiments was completely randomized (DIC) in a factorial scheme, according to the model:

$$y_{(ijk)} = \mu + \beta_{(i)} + \tau_{(j)} + \beta\tau_{(ij)} + \varepsilon_{(ijk)}$$

Where, $y_{(ijk)}$ Is the observed data in the, *ijk*; μ is a constant (general average); $\beta_{(i)}$, is the effect of the factor *i*; $\tau_{(j)}$, is the effect of the factor *j*; $\beta\tau_{(ij)}$, is the interaction between the factors β e τ; $\varepsilon_{(ijk)}$, is the experimental error of the plot *ijk*.

In order to evaluate the viability of pollen obtained from the upper and lower thirds in the panicles, DIC was used in a 10×2 factorial scheme (ten genotypes × two sections of the panicle) with four replications, in order to verify differences between the pollen fertility obtained from the third upper and lower panicles. In this experiment, the evaluation was performed on the same day of pollen collection.

In the evaluation of pollen viability during the storage time, a 4×6 factorial scheme (four genotypes × six storage periods) with four replications was applied in the DIC, in the second experiment, with the aim to verify the influence of storage time on fertility of the pollen obtained from the upper and lower third of the panicle, as well as knowing the period in which they remain fertile. In this experiment, the first evaluation was performed on the day of collection of the pollens and the other evaluations were performed every 10 days until the 50th day of storage (DA).

Statistical analyses

The mean values of the pollen viability of the genotypes were used for the analysis of variance, while the average values of the pollen fertility obtained from the upper third and inferior of the panicle were used for the regression analyzes.

Transformations and analyzes of variance were performed using statistical software SAS (2002) system version 9.0. The regression analysis and the Tukey test at 5% probability were performed using the statistical software SISVAR (Ferreira, 2011).

RESULTS AND DISCUSSION

The analysis of variance showed a significant difference at 1% probability level by the *F* test for the genotype (G), panicle (S) and G×S interaction sources, according to the results in Table 2. According to Gomes (1990), the coefficient of variation observed in Table 2 is considered medium, indicating good experimental precision. Due to

Table 3. Analysis of genotype x from the split section of the upper and lower third of the panicle of sugarcane for pollen viability.

Treatment	Panicle section	
	Lower (%)	Upper (%)
G2	87,3325 a A	70,2975 ab B
RB813804	84,0600 ab A	45,3825 cd B
RB931011	81,4550 ab A	51,7775 abcd B
G10	80,6625 ab A	73,0275 a A
RB002504	71,6925 abc A	65,9500 abc A
RB872552	62,4275 bcd A	68,7550 ab A
RB867515	54,8000 cd A	48,8000 bcd A
G3	48,2700 d A	57,7225 abcd A
RB863129	46,7450 d B	72,8250 a A
G9	41,3000 d A	41,1625 d A
Overall average	65.8745	59.5700

DMS between genotypes = 23.24%; DMS between sections panicle = 14.15%.
Means followed by same letter do not differ by Tukey's test at 5% probability. Lower case letters distinguish the source of variation and genotype capital letters distinguish the source of variation section of the panicle. Least Significant Difference (LSD).

the significant result for G×S interaction, we performed the analysis of variance and applied the Tukey test at 5% of probability (Table 3).

In the lower third of the panicle, genotypes G2, RB813804, RB931011, G10 and RB002504 did not differ statistically (Table 3). However, G2 access differed significantly from genotypes RB872552, RB867515, G3, RB863129 and G9. In the upper third of the panicle, the genotypes G10, RB863129, G2, RB872552, RB002504, G3 and RB931011 showed no statistical differences between them. However, accesses G10 and RB863129 differed significantly from genotypes RB867515, RB813804 and G9, which exhibited the lowest averages. The range of pollen viability observed in the lower third of the panicle among the genotypes studied was 45.07%, while the variation observed in the upper third of the panicle was 31.87%. The distinct statistical differences between the genotypes of each section of the panicle, as well as the different amplitudes of variation of the pollen viability observed, are due both to the difference between accessions and to the influence of the environment on the expression of the characteristic. It is believed that the observed variations are predominantly genetic, since the environmental conditions in the EFCCD are considered ideal for flowering and maintenance of the pollen viability of sugarcane, presenting temperatures ranging between 18 and 32°C according to Araldi et al. (2010), photoperiod ranging from 12 h 30 min to 12 h 55 min and relative humidity above 67% according to Melloni (2012). However, it can be observed that the variation in the lower third of the panicle was higher than that of the upper third, indicating that the basal region of the inflorescence may be more influenced by the environment. This conclusion corroborates the results obtained by Amaral et al. (2012), who recommend that pollen collection be performed on panicles that present the upper third in a thesis to avoid dehydration of the pollen by exposure to temperature variation.

Table 3 shows that there was a significant difference between the lower and upper thirds of the panicle of genotypes G2, RB813804, RB931011 and RB863129. The genotypes G2, RB813804 and RB931011 showed higher pollen viability in the lower third of the inflorescence, while the RB863129 access was more viable in the upper third of the panicle. The inflorescence sections of genotypes G10, RB002504, RB872552, RB867515, G3 and G9 did not differ statistically. In general, the inflorescences of the evaluated genotypes present greater viability in the lower third (Table 3). These considerations suggest that the collection of pollen for conservation can be performed throughout the inflorescence, not only in the upper third of the panicle as suggested by Amaral et al. (2012).

Regarding pollen viability during the storage time, the analysis of variance showed a significant difference, at the 1% probability level by *F* test, for the sources of variation G, DA and for interaction G×DA, according to the results in Table 4. The coefficient of variation of 10.96 observed is considered medium, indicating good experimental accuracy, according to Gomes (1990). Due to significant differences for the G×DA interaction, the sources of variation in the analysis of variance were deployed and Tukey's test was applied at 5% probability (Table 5).

There was no statistically significant difference between the varieties studied on the day of collection (0 DA), by the Tukey test at 5% probability (Table 5). According to Gómez (1962), all genotypes presented initial pollen

Table 4. Analysis of variance for the average values of pollen viability obtained from the panicle.

Variation sources	Degrees of freedom	Sum of squares	Mean square
Genotypes (G)	3	2.3172	0.7724 **
Days storage (DA)	5	1.9711	0.3942 **
Genotype × days storage (G × DA)	15	0.4937	0.0329 **
Residue	72	0.3723	0.0052
Total fixed	95	5.1543	

Coefficient of Variation (%) = 10.96.
**Significant at 1% probability by the F test.

Table 5. Analysis of the unfolding of genotype x days of storage with Tukey test at 5% probability level for the mean values of pollen viability panicle.

Genotypes	Storage periods (days)					
	0	10	20	30	40	50
RB813804	64,72 a A	50,62 a AB	41,61 a B	39,39 a B	37,24 ab BC	24,52 b C
RB931011	68,82 a A	60,71 a AB	48,86 a BC	48,84 a BC	47,40 a BC	44,72 a C
RB863129	59,79 a A	49,63 a AB	41,61 a BC	38,15 a BC	32,82 b CD	22,47 b D
RB872552	65,59 a A	14,05 b B	09,66 b BC	08,88 b BC	08,15 c BC	03,15 cC
Average	64.73	43.75	35.44	33.82	31.40	23.72

Means followed by same letter do not differ by Tukey's test at 5% probability. Lower case letters distinguish the source of variation and genotype capital letters distinguish the source of variation days of storage.

viability considered intermediate, indicating that these genotypes can be used as male in artificial crosses (McIntyre and Jackson, 2001). In the periods of 10, 20 and 30 DA, the genotypes RB931011, RB813804 and RB863129 did not present significant differences between them; however, they differed from RB872552, which showed the lowest values of pollen viability. At 40 DA, the best results were observed for RB813804 and RB931011. The RB863129 genotype did not differ statistically from RB813804, but was significantly different from RB872552 that once again presented a lower result than the other varieties. At 50, the genotype RB931011 differed from the others and presented the highest average of polynomial viability, and the variety RB872552 again showed the lowest mean.

The mean pollen viability of RB813804, RB931011 and RB863129 genotypes in the final evaluation was 24.52, 44.72 and 22.47%, respectively. According to McIntyre and Jackson (2001), parents with viability greater than 20% can be used as males for by-parental crosses. According to Buitink and Leprince (2004), the stored pollen grains remain viable, possibly due to the occurrence of vitreous (semi-solid state, highly viscous liquid) in the cytoplasm.

Each genotype showed a distinct behavior regarding loss of pollen viability during the storage time. In the initial evaluation, the pollen viability of genotype RB813804 was similar to the evaluation of 10 DA. In the period from 10 AD to 40 AD the viability of the pollen ranged from 50.62 to 37.24%, respectively, without showing significant differences by the Tukey test at 5% probability. In the last evaluation, at 50 DA, it reached 24.52% (Table 5), this value is at the threshold considered satisfactory for the use of male parents in artificial crosses (McIntyre and Jackson, 2001).

Table 5 shows that the genotype RB931011 presents initial pollen viability of 68.82% and gradually the viability declined to 44.72% at 50 DA. The mean value observed in the final evaluation may be considered intermediate, and may also be recommended as a male parent in artificial crosses (McIntyre and Jackson, 2001).

The genotype RB863129 presented initial polynic viability of 59.79%, which did not statistically vary from that obtained at 10 DA. In the subsequent periods, the fertility of the pollen fell considerably, reaching 22.4% (Table 5). The final viability is considered satisfactory and remains at the threshold of the reference value proposed by McIntyre and Jackson (2001).

On the day of pollen collection, genotype RB872552 presented initial viability of 65.59%, dropping drastically to 3.15% at 50 DA of storage. This access showed the highest variation among all evaluated genotypes, with significant differences observed in the periods studied. The low final pollen viability suggests that the genotype is not indicated for end of conservation.

From Table 5, it is observed that there was a significant difference between the viability of the genotypes in the final evaluation, which requires another evaluation at the

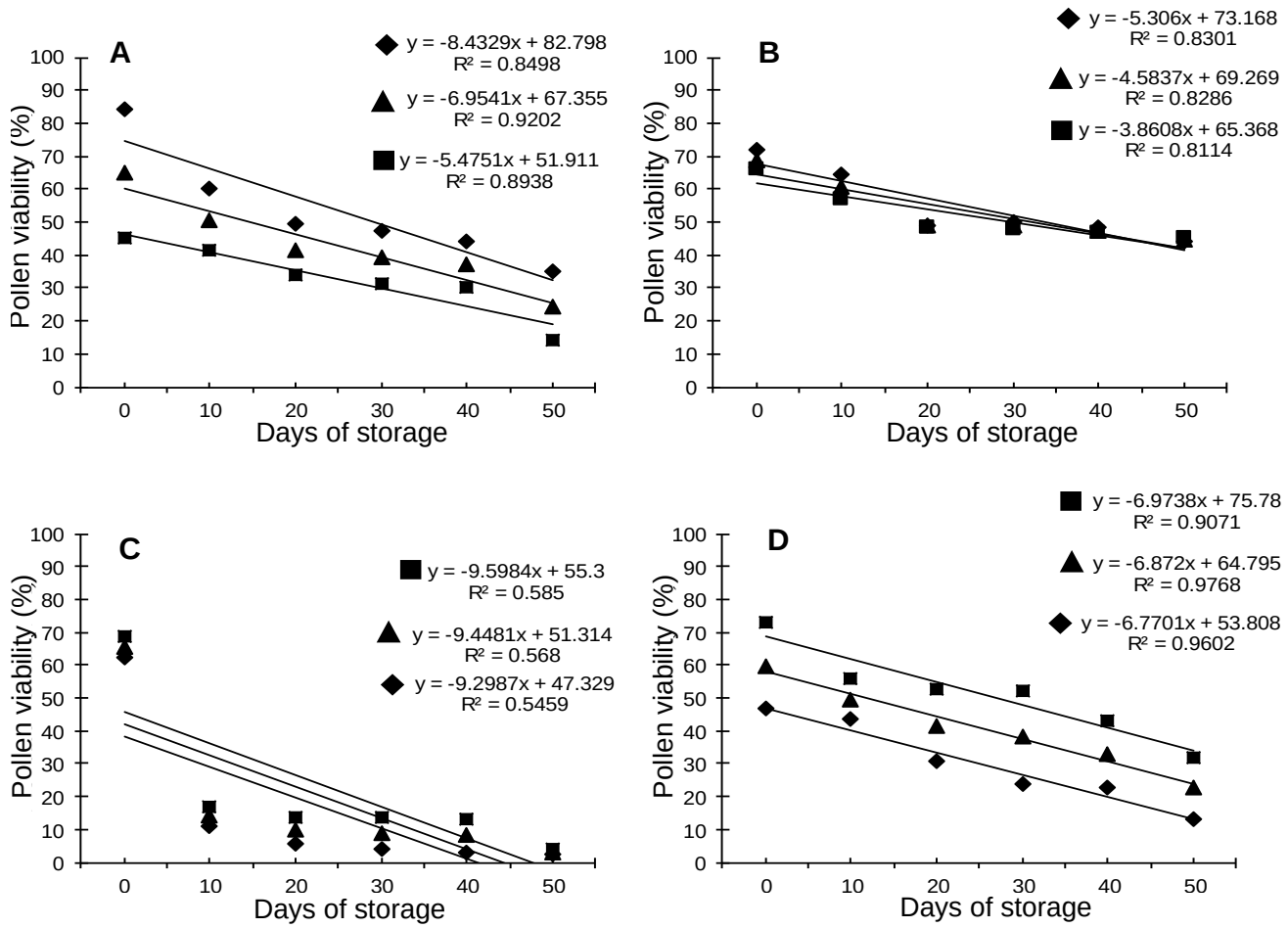

Figure 1. Values obtained from regression of the upper section (■), lower (♦) and Average (▲) for pollen viability loss during the storage time: "A" genotype RB813804, "B" genotype RB931011, "C" genotype RB872552 and "D" genotype RB863129.

moment of pollination, agreeing with the results presented by Hanna (1994), Tighe (2004) and Amaral et al. (2012).

The regression analysis verified a significant difference at 1% probability level for all variables studied, according to Figure 1. All evaluated genotypes showed a linear behavior in relation to loss of pollen viability in the upper and lower thirds of the panicle, as well as in the average viability. The coefficients of determination (R^2) obtained were higher than 80% for the varieties RB813804, RB863129 and RB931011, which can be observed in Figures 1A, B and D, respectively; indicating that the regression analysis was efficient to elucidate the biological phenomenon of the loss of pollen viability during the pollen storage time obtained from the upper and lower thirds and the average viability.

The marked decline of pollen viability observed in genotype RB872552 (Figure 1C), may be associated with the formation of ice crystals in the intracellular environment, because according to Benson (2008), such condition can cause physical ruptures and mechanical injuries, leading to loss of cellular components. However, since the pollen grains have been dehydrated, it is believed that this is a genetically determined characteristic, which is an intrinsic factor of the variety, making conservation impossible.

Accessions RB813804, RB863129 and RB931011 demonstrated significant loss of pollen viability after 20 days of storage, but presented satisfactory values in the final evaluation, indicating that these can be used for the purpose of pollen conservation. According to Song and Tachibana (2007), under controlled conditions, loss of pollen viability is often associated with the existence or absence of tolerance to desiccation of pollen. Studies indicate that deterioration of pollen during aging involves loss of intracellular integrity, decreased cytochrome oxidase enzyme activity, free radical accumulation, esterification and lipid peroxidation of the membrane, leading to increased loss of components after rehydration (Taylor and Hepler, 1997). According to Wolkers and Hoekstra (1995), membrane damage caused by pollen aging is not associated with protein denaturation.

However, loss of pollen viability may be associated with the reduction of pollen protein synthesis after rehydration, an imperative factor for the start of the pollen tube emission (Hiscock and Allen, 2008).

Conclusion

Accessions G2, RB813804 and RB931011 presented higher pollen viability in the lower third of the inflorescence. While the variety RB863129 presented greater viability of the pollen in the upper third.

The pollen conservation technique of sugarcane at -18°C is efficient to maintain the pollen viability of genotypes RB813804, RB863129 and RB931011.

The pollen conservation methodology allows crosses between parents with asynchronous flowering and increased possibilities of exploring the genetic variability available in the ABG.

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