An Acad Bras Cienc (2021) 93(3): e20190901 DOI 10.1590/0001-3765202120190901
Anais da Academia Brasileira de Ciências | *Annals of the Brazilian Academy of Sciences*
Printed ISSN 0001-3765 I Online ISSN 1678-2690
www.scielo.br/aabc | www.fb.com/aabcjournal

CELLULAR AND MOLECULAR BIOLOGY

In vitro storage of sweet passion fruit seeds as an innovation conservation alternative

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Abstract: Sweet passion fruit (*Passiflora alata* Curtis) is a species native to Brazil that is economically important for fruit consumption and can also be used as an ornamental plant. The objective of this work was to evaluate different forms of storing *Passiflora alata* seeds for the purpose of preserving accessions in germplasm banks. Three experiments were performed. In the first, the seeds without aril were stored for two periods (one and three years) in plastic bags and in *in vitro* culture medium at two temperatures. In the second, the seeds with and without aril were stored for one year in plastic boxes and *in vitro* culture medium at two temperatures. In the third, the seeds were kept for two years in different *in vitro* culture media. The seeds quickly lost viability when stored for one year conventionally in a cold chamber with 25% emergence, but remained viable when were stored *in vitro* with 90% emergence. Therefore, the use of *in vitro* culture medium is an innovative way to preserve sweet passion fruit seeds at 25 °C. The seeds of the *Passiflora alata* evaluated should be considered recalcitrant.

Key words: *Passiflora alata*, seed germination, genetic resources, germplasm, *ex situ* conservation.

INTRODUCTION

Sweet passion fruit (*Passiflora alata* Curtis) is a species native of the Amazon region of Brazil, Peru and Colombia that is economically important for fruit consumption and can also be used as an ornamental plant (Figure 1). Furthermore, the leaves have sedative and tranquilizing properties (Provensi et al. 2008, Klein et al. 2014) and are a good source of antioxidant, anti-inflammatory and potentially of anti-diabetic compounds (Colomeu et al. 2014, Figueiredo et al. 2016). It has wide distribution in Brazil, from the states of Amazonas in the north to Rio Grande do Sul in the south (Bernacci et al. 2015).

Passion fruit plants can be propagated from seeds or asexually by grafting, cuttings or tissue culture. However, propagation from seeds is most often used by growers and research institutions. Therefore, it is fundamental to know the germinative potential and conservation capacity of its seeds, to improve the production of seedlings and the management of germplasm banks.

The storage of passion fruit seeds can be a secure and inexpensive way to maintain germplasm banks (Brütting et al. 2013, Posada et al. 2014), possibly favoring the physiological maturation of the seeds. However, problems with germination and storage of seeds are very common with the genus *Passiflora*, even the sour passion fruit (Meletti 2011, Osipi et al. 2011), imposing a limiting factor on genetic improvement programs.

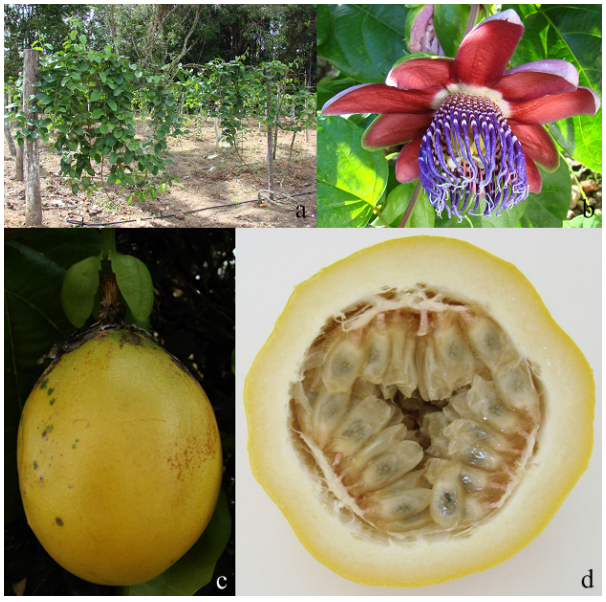

Figure 1. ***Passiflora alata*****: a) field plant, b) flower, c) fruit in the plant and d) cut fruit.**

Some passion fruit species are subject to a significant loss of seed viability during storage, which can be influenced by type of storage, ambient conditions and time frame (Pádua et al. 2011, Posada et al. 2014, Santos et al. 2016). According to Junghans (2015), more research is necessary regarding storage of seeds of *P. alata*, because they can lose viability after only one month of storage at room temperature or under refrigeration.

An innovative alternative method to preserve seeds of *Passiflora* species that quickly lose viability when kept under refrigeration is *in vitro* conservation. This method has been successfully applied to improve the preservation of species for vegetative propagation, under conditions that limit growth, as a way to preserve the germplasm for short and medium time intervals (Souza et al. 2013). The innovation would be to use it to preserve seeds by tailoring the composition of the culture medium, including the addition of germination inhibitors.

The objective of this study was to evaluate different ways of storing sweet passion fruit seeds (Figure 2) for conservation of accessions in germplasm banks and also for grading in the production of seedlings.

MATERIALS AND METHODS

Plant material and culture medium

The experiments were conducted in the Tissue Culture Laboratory, the Seed Conservation and Technology Laboratory and a greenhouse of the Embrapa Cassava and Fruits research unit (Embrapa Mandioca e Fruticultura), in the municipality of Cruz das Almas, Bahia (12° 39' 25" S, 39° 07' 27" W, 226 m). The climate in the region is BSa according to the Köppen classification, with average annual potential evapotranspiration greater than average yearly rainfall, dry season in the summer, average temperature above 22 °C in the hottest month and average yearly relative humidity of about 80%.

Three complementary experiments were performed. In the first, we verified the possibility of *in vitro* seed conservation for up to three years of storage, using as control the storage of seeds in the conventional form, in which the seeds

Figure 2. Type of storage of passion fruit seeds: a) conventional method and b) *in vitro* culture medium.

are packed in a transparent plastic bag (0.05 mm thickness) and kept in a refrigerator. In the second, we studied the effect of the presence of aril, and in the third, if the composition of the *in vitro* culture media would affect *in vitro* seed conservation.

The *P. alata* seeds were obtained from fully-ripe fruits harvested from the experimental field of the Active Germplasm Bank of *Passiflora* of Embrapa Cassava and Fruits. After harvested, the fruits were washed and then flame sterilized three times in a laminar flow cabinet. After sterilization, the fruits were cut and the seeds were extracted and pooled.

The culture medium for storage was MS (normal concentration of the medium of Murashige & Skoog 1962) or ½MS (half the normal concentration of salts in the MS medium), in both cases with normal concentration of vitamins, supplemented with 30 g/L of sucrose and solidified with 2.6 g/L of Phytagel, pH 5.8, with or without addition of gibberellic acid (GA_3) and activated charcoal. Aliquots (25 mL) of the various culture media were poured into round flasks with plastic screw-on lids, which were autoclaved at 121 °C (1.05 Kg/cm^2) for 20 minutes.

Experiment 1 - Seeds without aril stored for two periods (one year and three years) in plastic bags and in vitro culture medium (accession BGP438)

In one portion of the seeds the aril was removed with a scalpel and tweezers before inoculation in ½MS medium for the storage test. In the other portion, the aril was removed by washing in a plastic sieve. Then the excess water was removed by placing the seeds on paper. After that, a portion of the seeds were used in the initial test of seedling emergence from recently harvested seeds. The other portion was storage in a transparent plastic bag with thickness of 0.05 mm.

The storage of the seeds was conducted for two periods (one and three years) under *in vitro* and *ex vitro* conditions, at constant temperature (25 °C) and under refrigeration (2 ± 3 °C).

Experiment 2 - Seeds with and without aril, stored for two periods (one month and one year), in plastic bags and in vitro culture medium (accession BGP162)

In one portion of the seeds the aril was removed with a scalpel and tweezers before inoculation in MS medium for the storage test. In another portion, the aril was not removed from the seeds, which were stored in round flasks with screw-on lids. Finally, in the third portion of the seeds, the aril was removed by washing in a plastic sieve, the excess water was removed by placing the seeds on paper, and the seeds were stored in round flasks with screw-on lids.

The storage of the seeds with and without aril were conducted for two periods (one month and one year) under *in vitro* and *ex vitro* conditions, at constant temperature (25 °C) and under refrigeration (2 ± 3 °C).

Experiment 3 - Seeds stored for two years in different culture media (accession BGP004)

The aril was removed from all the seeds with a scalpel and tweezers and then the seeds were inoculated on different *in vitro* culture media for storage. The *in vitro* culture media used were:

- **Medium A**: ½MS (half the regular concentration of salts in MS medium), normal concentration of vitamins, supplemented with 30 g/L of sucrose and solidified with 2.6 g/L of Phytagel®, pH 5.8.
- **Medium B**: ½MS, normal concentration of vitamins, supplemented with 30 g/L of sucrose, 0.3 mg/L of GA_3, and solidified with 2.6 g/L of Phytagel, pH 5.8.

- **Medium C**: **medium A** supplemented with activated charcoal (2 g/L).
- **Medium D**: **medium B** supplemented with activated charcoal (2 g/L).

The storage of the seeds was conducted for two years in the various culture media, in a temperature-controlled chamber with alternate temperature of 20 and 30 °C (16 and 8 hours respectively), in the absence of light.

Water content and emergence tests

The water content of the seeds was calculated on a wet weight basis and it was estimated by averaging the weight loss of three subsamples of 10 seeds each placed in an oven at 105 °C (Brasil 2009). The emergence tests were conducted in a greenhouse for the recently harvested seeds and after storage of the seeds with four repetitions of 25 seeds each. The seeds were sown with depth of 1.0 cm in tubes with volume of 280 cm^3 containing vegetable substrate Vivatto®.

The evaluations of the three experiments were performed daily from sowing until the start of emergence of seedlings, and then with further evaluations every other day until the 30^{th} day after sowing. Seedlings with cotyledonary leaves above the substrate level were considered to have emerged.

Experimental design

The experimental design was completely randomized. The following seedling emergence variables were considered: first count, emergence percentage, mean emergence time, mean emergence rate, and synchrony. The mathematical expressions and interpretations of these variables are described by Ranal & Santana (2006).

All the variables were submitted to the Lilliefors test for normality ($p<0.01$) and the Bartlett test for homogeneity of variances, calculated with the Genes software (Cruz 2013). After confirming satisfaction of the statistical prerequisites, analysis of variance was applied along with comparison of the means by the F-test, Tukey and Scott-Knott test at 5% probability, with the Sisvar software (Ferreira 2011).

RESULTS AND DISCUSSION

The results obtained in the first two experiments showed that sweet passion fruit seeds are recalcitrant and do not tolerate water content in the seeds for storage equal to or lower than 14.6% in accordance with Hong & Ellis' definition (1996). The findings also corroborate the inability to maintain sweet passion fruit seeds in the conventional conditions used to store seeds of the majority of passion fruit species. The first two experiments also showed that storage of sweet passion fruit seeds *in vitro* was an excellent technique for maintaining seed viability. The third experiment showed that the composition of the culture medium did not affect seed conservation *in vitro*.

Experiment 1 - Seeds without aril stored for two periods (one year and three years) in plastic bags and *in vitro* culture medium (accession BGP438)

The water content of the recently harvested seeds was 21.7%, and after three years of storage it differed among the four storage methods, varying from 7.4% to 25.2% (Table I). Only the water content of the seeds stored *in vitro* at 25 °C remained near to that of the recently harvested seeds.

The best way of storing the sweet passion fruit seeds among the four methods tested was by *in vitro* culture at 25 °C (Table II). Under those conditions, it was possible to maintain the seed viability for three years with 78% emergence, a percentage similar to that of the recently

Table I. Water content percentage in the different storage conditions of *Passiflora alata* (BGP438), seeds at the start and end of storage.

Treatment	Water content of the seeds (%)
Recently harvested	21.7 b
3 years; *in vitro*; 25 °C	22.6 b
3 years; *in vitro*; 2 ± 3 °C	25.2 a
3 years; plastic bag; 25 °C	9.4 c
3 years; plastic bag; 2 ± 3 °C	7.4 d
CV (%)	1.87

Means followed by the same letter do not differ by the Tukey test at 5% probability.

harvested seeds (95%) and those stored for one year (90%). Although not statistically significant, there was a tendency for emergence to decline during the *in vitro* storage period. However, the seeds stored in *in vitro* conditions under refrigeration totally lost their viability after one year, probably because of freezing of the culture medium. For the seeds kept in plastic bags, the emergence was very low (25%), and this was only observed after one year of storage, only for the seeds kept under refrigeration.

Other authors also observed low seedling emergence percentage of sweet passion fruit after seed storage at low temperature. Santos et al. (2016) obtained 82% emergence of recently harvested *P. alata* seeds, but only 4% from seeds after storage for 11 months at 10 °C and 60% relative humidity, with a water content of 9.4%. Freitas et al. (2015) also obtained a low seedling emergence percentage for *P. alata* seeds, with maximum emergence of 25%, although they did not specify the length of the storage period before the emergence test.

Osipi & Nakagawa (2005a) stored *P. alata* seeds for up to one year under three conditions: within paper bags under uncontrolled ambient and in a dry chamber, and in a cold chamber with seeds kept in polyethylene bags. These authors did not observe any difference among the storage methods after six months, but after one year the best preservation was attained by the seeds stored in plastic bags at 10 °C, for which the water content was approximately 10%.

Table II. Average first count emergence percentage of seedlings 15 days after sowing (DAS) and emergence of seedlings 30 DAS, mean emergence time of seedlings (days), mean emergence rate and synchrony of emergence of seedlings 30 DAS in the different storage periods and conditions of *Passiflora alata* (BGP438) seeds. First count = FC, emergence = E, mean time = MT, *in vitro* = IV, plastic bag = PB.

Treatment	FC (%)	E (%)	MT (days)	Mean Rate	Synchrony
Recently harvested	30 b	95 a	18 b	0.055 b	0.115 b
1 year; IV; 25 °C	78 a	90 a	17 b	0.059 b	0.610 a
1 year; IV; 2 ± 3 °C	0	0	0	0	0
1 year; PB; 25 °C	0	0	0	0	0
1 year; PB; 2 ± 3 °C	0	25 b	18 b	0.032 b	0.125 b
3 years; IV; 25 °C	75 a	78 a	12 a	0.084 a	0.345 b
3 years; IV; 2 ± 3 °C	0	0	0	0	0
3 years; PB; 25 °C	0	0	0	0	0
3 years; PB; 2 ± 3 °C	0	0	0	0	0
CV (%)	17.11	10.61	6.94	7.19	36.28

Means followed by the same letter within a column do not differ by the Tukey test at 5% probability.

The emergence values obtained 15 days after sowing (first count) were higher for one and three years of storage than for the recently harvested seeds, suggesting the presence of dormancy, which may have been broken by the *in vitro* storage (Table II). The hypothesis of reduced dormancy is reinforced by the reduction of the mean emergence time from 18 days for recently harvested seeds to 12 days for seeds stored for three years, and also by the increase in the average emergence rate of the seeds kept for three years. In turn, the synchrony was better among the seeds stored for one year, probably due to the association of vigor with reduced dormancy.

Experiment 2 - Seeds with and without aril, stored for two periods (one month and one year), in plastic bags and *in vitro* culture medium (accession BGP162)

In experiment 2, the seeds' water content also varied among all the storage conditions, with the highest occurring under the *in vitro* conditions (34.5% and 25.9%) and the lowest under the *ex vitro* conditions, varying from 7.4% to 14.6% (Table III). Only the water content of the seeds kept *in vitro* at 25 °C remained similar to that of the recently harvested seeds.

The best storage method of the sweet passion fruit seeds was again *in vitro* culture, but unlike what happened in the first experiment, the seeds maintained under refrigeration presented a good seedling emergence percentage (Table IV). This result suggests that seeds kept *in vitro* under refrigeration are subject to uneven temperatures, depending on the location within the refrigerator, and can freeze and totally lose viability, as happened in the first experiment. After storage for one month, there was no difference in the emergence of seedlings from seeds stored *in vitro* kept under refrigeration or at 25 °C. After storage for one year, the emergence was highest (95%) for the seeds stored *in vitro* at 25 °C, in comparison with those kept in the refrigerator, with 68% seedling emergence. Again, this might have been caused by temperature differences inside the refrigerator. However, to confirm this theory it would be necessary to conduct new tests at 5 °C, without allowing the temperature to drop below zero, since this can cause the culture medium and seeds to freeze. The seedling emergence obtained after one year of storage at 25 °C (95%) was higher than for those stored for one month (78%), which can be attributed to the breakage of dormancy.

Among the *ex vitro* storage methods, even storage for only one month caused a large loss of vigor, including values of zero on the first count (Table IV). This was also revealed by the worse values of seedling emergence, mean emergence time, mean emergence rate and synchrony. For *ex vitro* storage for one month, it was best

Table III. Mean water content percentages of *Passiflora alata* (BGP162) seeds under different storage conditions and periods.

Treatment	Water content of the seeds (%)
Recently harvested	21.7 c
1 year; *in vitro*; without aril; 25 °C	25.9 b
1 year; *in vitro*; without aril; 2 ± 3 °C	34.5 a
1 year; *ex vitro*; without aril; 25 °C	9.4 f
1 year; *ex vitro*; without aril; 2 ± 3 °C	7.4 g
1 year; *ex vitro*; with aril; 25 °C	12.4 e
1 year; *ex vitro*; with aril; 2 ± 3 °C	14.6 d
CV (%)	2.33

Means followed by the same letter do not differ by the Tukey test at 5% probability.

Table IV. Mean percentages of first count of emergence of seedlings 15 days after sowing (DAS) and emergence of seedlings 30 DAS, mean emergence time (days), mean emergence rate and synchrony of seedling emergence 30 DAS under different storage conditions and periods of *Passiflora alata* (BGP162) seeds. First count = FC, emergence of seedlings = E, mean time = MT, *in vitro* = IV, *ex vitro* = EV, without aril = WOA, with aril = WA.

Treatment	FC (%)	E (%)	MT (days)	Mean Rate	Synchrony
1 month; IV; WOA; 25 °C	60 a	78 b	19 a	0.0590 b	0.3393 b
1 month; IV; WOA; 2 ± 3 °C	76 a	82 b	13 a	0.0803 a	0.5378 a
1 month; EV; WOA; 25 °C	0	0	0	0	0
1 month; EV; WOA; 2 ± 3 °C	0	11 d	62 c	0.0163 c	0.0803 c
1 month; EV; WA; 25 °C	0	47 c	43 b	0.0233 c	0.1493 c
1 month; EV; WA; 2 ± 3 °C	0	42 c	36 b	0.0303 c	0.2105 c
1 year; IV; WOA; 25 °C	85 a	95 a	11 a	0.0910 a	0.4170 b
1 year; IV; WOA; 2 ± 3 °C	58 a	68 b	13 a	0.0765 a	0.3415 b
1 year; EV; WOA; 25 °C	0	0	0	0	0
1 year; EV; WOA; 2 ± 3 °C	0	0	0	0	0
1 year; EV; WA; 25 °C	0	0	0	0	0
1 year; EV; WA; 2 ± 3 °C	0	0	0	0	0
CV (%)	22.43	16.93	18.45	23.12	28.62

Means followed by the same letter within a column belong to the same group by the Scott-Knott test at 5% probability.

to maintain the seeds with aril, regardless of temperature (refrigeration or 25 °C).

Experiment 3 - Seeds stored for two years in different culture media (accession BGP004)

The sweet passion fruit seeds maintained *in vitro* for two years, even in conditions that stimulated germination, such as addition of gibberellic acid and submission to an alternated temperature regime of 20 °C/30 °C, did not germinate under the *in vitro* conditions. Various researchers have reported that gibberellic acid (Ferreira et al. 2005, Ferrari et al. 2008, Santos et al. 2016) and temperature alternation of 20 °C/30 °C (Zucareli et al. 2003, Osipi & Nakagawa 2005b) stimulate germination of the seeds of *Passiflora* species, including sweet passion fruit.

However, after sowing the seeds conserved *in vitro* in the commercial substrate, the emergence percentage was high (88%), irrespective of the *in vitro* culture medium used for storage (Table V). There also was no difference in the vigor between the different culture media for storage, since the variables first count, mean time and mean rate of seedling emergence, which indicate seed vigor, did not present differences among the media.

Carvalho et al. (2012) also observed low *in vitro* germination percentage of *P. gibertii* seeds (10%), even with the addition of gibberellic acid to the culture medium. But when scarifying the outside of the seeds, they obtained a high *in vitro* germination percentage (91%). Similar results were obtained by Junghans et al. (2008), who reported low germination of intact seeds but higher germination when the tegument was partially removed from *P. gibertii* seeds, independently of the *in vitro* and *ex*

Table V. Mean percentages of first count of emergence of seedlings 15 days after sowing (DAS) and emergence of seedlings 30 DAS, mean emergence time (days), mean emergence rate and synchrony of seedling emergence 30 DAS after storage of *Passiflora alata* (BGP004) seeds for two years in different *in vitro* culture media. First count = FC, emergence = E, mean time = MT, MS medium with half concentration of salts = ½MS, gibberellic acid = GA_3, activated charcoal = AC.

Treatment	FC (%)	E (%)	MT (days)	Mean Rate	Synchrony
½MS	51 a	88 a	17 a	0.0602 a	0.2492 a
½MS + GA_3	46 a	88 a	16 a	0.0618 a	0.3336 a
½MS + AC	49 a	86 a	16 a	0.0624 a	0.2601 a
½MS + GA_3 + AC	38 a	88 a	17 a	0.0600 a	0.3568 a
CV (%)	24.99	9.62	3.74	3.73	25.63

Means followed by the same letter within column do not differ by the F-test at 5% probability.

vitro conditions. However, these latter authors reported that the *in vitro* germination was slower than in the *ex vitro* case.

CONCLUSIONS

1. The seeds of the sweet passion fruit accessions BGP438 and BGP162 evaluated are recalcitrant.

2. The preservation of sweet passion fruit accession BGP438 seeds in *in vitro* culture at temperature of 25 °C allows obtaining a high percentage of seedling emergence, even after three years of storage.

3. The conditions that cause breakage of dormancy of most seeds preserved *in vitro*, temperature alternation and application of gibberellic acid, are not sufficient to break the dormancy of the passion fruit accession BGP004 evaluated after being kept in *in vitro* culture medium.

Acknowledgments

The authors thank Fundação de Amparo à Pesquisa do Estado da Bahia (FAPESB) and Empresa Brasileira de Pesquisa Agropecuária (Embrapa) for financially supporting the research.

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How to cite

JUNGHANS TG, JESUS ON, SILVA JJ & FERREIRA MS. 2021. *In vitro* storage of sweet passion fruit seeds as an innovation conservation alternative. An Acad Bras Cienc 93: e20190901. DOI 10.1590/0001-3765202120190901.

Manuscript received on August 7, 2019;
accepted for publication on February 27, 2021

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T.G.J. guided all aspects of the research project, provided the analysis and interpretation of data and wrote and revised the manuscript. O.N.J. provided the genetic resources and did a critical paper review. J.J.S. and M.S.F. were involved in the performed of the experiments and collected data. All authors read and approved the final manuscript.