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Cryopreservation of *Arachis pintoi* (Leguminosae) seeds

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Summary

Studies on the storage of seeds of the diploid cytotype (2n=2x= 20 chromosomes) of *Arachis pintoi* (Leguminosae) were conducted to preserve germplasm for the long term in liquid nitrogen (-196°C). The best cryo-procedure comprised: desiccation of the seeds to 4% water content (14 days at 40°C) and, subsequently, direct cooling in nitrogen liquid. The cryotubes were then rapidly warmed in a water bath at 38°C. With this treatment, after 7 days in a germinator up to 90% of the seeds germinated.

Introduction

The genus *Arachis* belongs to the family Leguminosae, which include about 70 species, all of them are natives of South America. The most interesting among them, from the point of view of economic importance is peanut or groundnut, *Arachis hypogaea* L., (Krapovickas and Gregory, 1994). *Arachis pintoi* Krapov. & W.C. Greg. (common name: perennial peanut or pinto peanut), is a novel and important forage legume in tropical and subtropical areas of the world (Pizarro and Rincón, 1994; Argel and Ramírez, 1996). The diploid cytotype, 2n=2x=20 chromosomes, (Fernández and Krapovickas, 1994) produces relatively few seeds and, like *A. hypogaea* (Navarro *et al.,* 1989), shows high loss of viability after 10 month of storage (Ferguson, 1993). Consequently, for the conservation of this germplasm it is necessary to maintain collections of plants in the field with the possible risk of destruction by natural calamities, pests and diseases (Withers and Engelmann, 1998). Recently *in vitro* storage of shoot tips of the species was developed although this approach only permits preservation of the germoplasm for 1 year (Rey and Mroginski, 2005). The cryopreservation of seeds, using liquid nitrogen (LN) (-196°C), may overcome this problem because at this sub zero temperature most of the metabolic activities are brought to a standstill and consequently the deterioration of the seeds does not take place. In addition the approach also has the advantage of low maintenance costs for long-term storage of germplasm (Stanwood, 1985). Although cryopreservation for long term germplasm conservation has been developed using seeds of several plant species (Pence, 1991; Kameswara Rao, 2004; Sakai and Engelmann, 2007), there are no studies on the effect of cryopreservation on seeds of *Arachis pintoi.* The present work aimed at exploring the feasibility of cryopreservation of *Arachis pintoi* seeds using a vitrification method.

Materials and methods

Plant materials were obtained from adult field-grown plants of the cytotype diploid (2n=2x=20 chromosomes) of *Arachis pintoi* (herbarium specimen Gregory and Krapovickas 12787, deposited in CTES). In order to break the dormancy, the seeds were treated for 14 days at 40°C (Ferguson, 1993). Subsequently, the seeds (4% water content) were subjected to the treatments (100 seeds per treatment) detailed in table 1. While the seeds of the control (treatment 1) went directly to the germinator, the seeds subjected to other treatments (2-12) were placed in test tubes of 10 ml (10 seeds/tube) with different vitrification solutions (consisting of mixtures of various concentrations of sucrose and glycerol) and then placed in the germinators (treatments 4, 7 and 10). Other treatments involved exposing the material to the vitrification solutions then freezing the material directly in LN at -196°C (- Rapid freezing method - treatments 2, 5, 8 and 11) or the material was frozen slowly - 1°C/min up to -30°C (using a Controlled Rate Freezing System Model 9000 from Gordinier Electronics Inc., USA), then placed in LN at -196°C (-Slow freezing method - treatments 3,6, 9 and 12). The vitrification solutions consisted of sucrose (15, 25 or 50%) and glycerol (15, 25 or 50%). After an hour in the frozen state the cryotubes were rapidly warmed in a water bath at 38°C and the seeds were carefully washed with distilled water (5 times), to eliminate the vitrification solutions, and then sowed in germinators in a acclimatized room at 27 ± 2°C with a photoperiod of 14h (light intensity: 15 µmol $m^{-2}s^{-1}$).

Table 1. Effects of the vitrification solutions on the germination of cryopreserved seeds of *Arachis pintoi*.

Treatment	Vitrification solutions (%)		Freezing		Seed germination (% ± SE) after rewarming
	Sucrose +	Glycerol	Rapid	Slow	
1	0	0	---	---	81 ± 7
2	0	0	X[a)]	---	70 ± 5
3	0	0	---	X	74 ± 2
4	15	15	---	---	80 ± 6
5	15	15	X	---	77 ± 3
6	15	15	---	X	77 ± 3
7	25	25	---	---	83 ± 2
8	25	25	X	---	83 ± 2
9	25	25	---	X	78 ± 5
10	50	50	---	---	80 ± 5
11	50	50	X	---	83 ± 3
12	50	50	---	X	78 ± 4

a) Method tested

The germinators were transparent plastic boxes of 16 cm length × 12 cm width × 4.5 cm height. The seeds (10 seeds / germinator) were sown on a 5mm layer of cotton that was soaked with distilled water (figure 1). After 7 days the germination percentage was determined**.** Emergence of a normal root from the seed was used as the criterion for the estimation of the germination rate. The experiments were replicated 3 times. All data were subjected to analysis of variance (ANOVA) and comparisons of means were made with Tukey's Multiple Comparison Test at $P<0.05$.

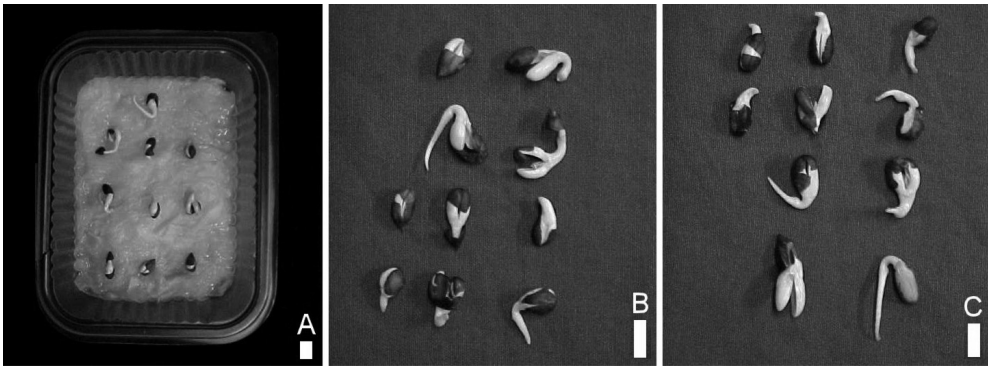

Figure 1. Germination of cryopreserved seeds of *Arachis pintoi* after 7 days in germinator. A) control (treatment 1 of table 1). B) seeds from treatment 8. C) Seeds from treatment 3.

Results and discussion

All of the treatments employed permitted high percentages of seed germination (table 1). Survival of controls (non-cooled) seeds of *Arachis pintoi* was high, ±77% of germination (figure 1), whilst after freezing and thawing the germination ranged between 70 and 90%. However, the results achieved with the various treatments did not show significant differences from the controls. These results, on the one hand, confirmed the effectiveness, of the pre-treatment with high temperatures, to break the dormancy of the seeds of *A. pintoi*, as suggested by Ferguson (1993). On the other hand, they showed that these seeds did not lose viability when they were desiccated and subsequently stored at -196°C and that there was no significant effect of the various storage methods (rapid or slow freezing) on seed germination. Furthermore, the results show the ineffectiveness of the presently used vitrification solutions as cryoprotecting substances. In this respect, *A. pintoi* seeds appear to be similar to other plant species where liquid nitrogen exposure was found to be non-detrimental to seed germination. In this sense, Pence (1991) found that, working with 63 genera of native plants of Ohio, in 49 genera the direct exposure of the seeds to liquid nitrogen did not affect germination. Similar results were reported with seeds of various plant species of Australia (Touchell and Dixon, 1993), Spain (Gonzalez Benito *et al.,* 1998) and India (Decruse *et al.*, 1999). In our laboratory, 85% of seeds of Australian cedar (*Toona ciliata*) stored directly at -196°C germinated (Scocchi *et al.*, 2004).

As a consequence of the results shown in table 1 the best procedure for cryopreservation of seeds of *Arachis pintoi* comprised: desiccation to 4% water content (14 days at 40°C), direct cooling in nitrogen liquid and rewarming in a water bath at 38°C. With this treatment, after 7 days in a germinator the seeds germinated.

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