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Additional Information

Abstract

Bituminaria bituminosa (L.) C.H. Stirton is a drought tolerant, perennial legume pasture species and a source of pharmaceutical compounds. *Bituminaria* breeding programs aim to develop and conserve hybrids with desirable traits such as high forage quality, tolerance to biotic or abiotic stresses, and high contents of furanocoumarins. In this work we present a cryopreservation study of different *B. bituminosa* accessions: two varieties and eight intervarietal hybrids resulting from crosses between the three botanical varieties: var. *bituminosa*, var. *crassiuscula*, and var. *albomarginata.* No previous work on cryopreservation of *Bituminaria* species has been reported. We applied the ultra-fast cooling method, using droplet vitrification on aluminum foil strips. First, we investigated the PVS2 toxicity and cryopreservation damage in two genotypes, comparing three PVS2 treatments and two culture media. An incubation of 30 min in PVS2 resulted in regeneration rates after cryopreservation higher than 80%. The MS medium was selected for optimal meristem outgrowth, in order to avoid the prominent callus formation that was observed in the presence of BAP. These conditions were subsequently used to cryopreserve eight other genotypes. The results were highly variable; 45 days after cryopreservation, survival ranged between 22 and 98% while regeneration ranged between 0 and 96%, depending on the accession. A significant and positive correlation was observed between survival and regeneration. At 90 days post culture plantlets could be recovered from cryopreserved explants of all genotypes. This study shows that the droplet vitrification method is promising for the cryopreservation of eight of the 10 genotypes assayed and the method can thus be applied to develop a cryobank of *B. bituminosa*.

Keywords

Psoralea bituminosa; *var*. Albomarginata; *var*. Crassiuscula; tedera; droplet vitrification

Abbreviations

Me2SO: Dimethyl sulfoxide

MS: Murashige and Skoog basal medium (1962)

Dpc: Days post culture

Introduction

Bituminaria bituminosa (L.) C.H. Stirton (syn. *Psoralea bituminosa* L., Fabaceae, *Psoraleae*; Stirton, 1981) is a multipurpose perennial legume widely distributed in the Mediterranean Basin and Macaronesia. A large diversity exists in the Canary Islands with three botanical varieties described and other ecotypes under study. They are found in habitats ranging from the coastal semiarid areas of Lanzarote Island with an annual rainfall of 150 to 300 mm (var. *albomarginata*) to the high elevation subhumid area of Tenerife with up to 500 mm rainfall (var. *crassiuscula*). The third, var. *bituminosa,* has a wider adaptation across the Canary Islands, with significant differences among biotypes [15], and is also widely distributed in the Mediterranean basin [13,14,25].

The interest in *B. bituminosa* stems from its value as a drought tolerant pasture species [27] and as a potential source of pharmaceutical compounds, especially furanocoumarins (FCs) such as psoralen and angelicin. Psoralens are widely used to treat human skin diseases and for their antimicrobial activity and anti-HIV effects [7,10]. Angelicin has calmative, sedative, and anti-convulsant activities and is used for the treatment of thalassemia [US Patent No: US2006/0111433(A1)]. Other secondary metabolites of interest synthesized by *B. bituminosa* are the pterocarpans, which have anti-proliferative, estrogenic, hepatic-protective, anti-allergy, anti-inflammatory, apoptotic, and anti-tumor activities [12,24].

At the IMIDA Institute (Instituto Murciano de Investigación y Desarrollo Agrario y Alimentario, Murcia, Spain), breeding is executed to obtain lines or hybrids with desirable traits such as high forage quality, tolerance of biotic or abiotic stresses, and high content of FCs. When selecting for high FC content, the breeding program focuses on one single FC, to facilitate its extraction. For instance, the hybrid 'Bullas-La Perdíz' synthesizes more psoralen than angelicin while 'Calnegre' has a high angelicin: psolaren ratio. Also, SSR markers have been developed for genetic studies [21] and protocols for the micropropagation and regeneration of accessions have been described [22,23].

Cryopreservation represents a secure and sustainable method for the long-term conservation of germplasm. This technique is very convenient for materials of interest which are difficult to reproduce by seeds, such as hybrids, species that produce recalcitrant (=badly storable) seed, or materials selected for specific plant traits or characteristics that cannot be maintained by sexual propagation [18,1]. The aim of this work was to study the cryopreservation of *B. bituminosa* and develop a protocol for the cryopreservation of valuable *B. bituminosa* clones and intervarietal hybrids obtained from breeding

75 programs. Because no previous work on cryopreservation has been carried out with this species, we
76 assayed the droplet vitrification method. This method combines classical vitrification [29] and the droplet
77 freezing method first described for potato cryopreservation [31]. Droplet vitrification is now successfully
78 used for a wide variety of plant species like yam [8], banana [19], olive [30], pelargonium [4], thyme [9],
79 and avocado [5].

80

81 **Materials and methods**

82 *Plant material and culture conditions*

83 Two clones of *B. bituminosa,* var. *bituminosa* Calnegre (CN) and Llano del Beal (LB), and eight
84 intervarietal hybrids resulting from crosses between *B. bituminosa* var. *bituminosa* (either Mediterranean
85 or Canarian) and the other two *B. bituminosa* varieties (*albomarginata* and *crassiuscula*) were used as
86 sources of explants (Table 1). The origin and characteristics of the breeding materials, collected or
87 developed in the IMIDA, are described in Table 1.

88 Plants were established *in vitro* according to [22], micropropagated, and subsequently used as
89 sources of meristems. For micropropagation we used half-strength MS medium [17] supplemented with
90 active charcoal (0.25%), 10 µM indole-acetic acid (IAA), and 1 µM gibberellic acid (GA_3), and solidified
91 with 0.8% American Bacteriological agar (Pronadisa; Laboratorios Conda, Madrid, Spain). Unless stated
92 otherwise, the pH of the media was adjusted to 5.8 - before sterilization by autoclaving at 0.1 MPa and
93 121 ºC for 20 min. Plants were cultured under standard conditions: 16-h photoperiod with cool white light
94 (3000 lx) provided by Sylvania cool white F37t8/CW fluorescent lamps (90 µm m^{-2} s^{-1}), at 26 ºC ± 2 ºC.
95 Individual axillary meristems excised from *in vitro* micropropagated plants (5-8 cm height) were used in
96 all experiments. Meristems were excised under a binocular microscope, under aseptic conditions. Leaves
97 were removed with a scalpel; afterwards, stem segments of about 1-2 mm in size - comprising the
98 meristem (0.3-0.5 mm) - were used.

99 *Cryopreservation method and solutions*

100 We applied the ultra-fast cooling method using droplet vitrification on aluminum foil strips, as
101 described by Panis [20]. Three filter sterilized (0.22 µm SARTORIUS, Madrid, Spain) solutions were

102 used: (1) the Loading Solution (LS), composed of 2 M glycerol and 0.4 M sucrose; (2) the cryoprotective
103 vitrification solution PVS2, consisting of 3.26 M (30%) glycerol, 2.42 M (15%) ethylene glycol, 1.9 M
104 (15%) Me2SO, and 0.4 M sucrose, and (3) the recovering solution (RS), that contained 1.2 M sucrose. All
105 three solutions were dissolved in MS medium and the pH adjusted to 5.8.

106

107 *Cryopreservation experiments*

108

109 *Determination of PVS2 toxicity and freezing damage*

110

111 Meristems of genotypes CN and P6 were cultured on MS03 [MS salts including vitamins, 0.3 M
112 sucrose, 0.7% Plant Agar (Duchefa, The Netherlands)] for 1 day in the dark (preculture) before transfer to
113 30-ml polystyrene containers with approximately 10 ml LS. After 20 min at room temperature, the LS
114 was replaced by ice-cooled PVS2 at 0 ºC for four incubation periods: 0 min (PSV-0), 10 min (PVS-10),
115 30 min (PVS-30), and 50 min (PVS-50). For each PVS treatment, four Petri dishes with 8-10 meristems
116 of each genotype were treated. Half of the samples per PVS treatment were immersed in liquid nitrogen
117 and stored there for 1 hour (cryopreserved; C) while the other half were placed directly in RS (Non-
118 cryopreserved; NC). Following re-warming, the PVS2 was removed and replaced by RS. In both cases,
119 the meristems remained in RS for 15 min, followed by transfer to MS03 medium and incubation in
120 darkness for 1 day. After this period, half of the samples of each treatment were cultured on solid MS
121 medium [MS salts including vitamins, 0.15% sucrose, 0.7% Plant Agar] or on MS supplemented with
122 2.22 μM 6-benzyl-aminopurine (BAP) (MS BA medium). Meristems that were not incubated with PVS2
123 were used as controls. Two repetitions per treatment were performed, each consisting of two Petri dishes.
124 All cultures were kept in darkness at 25 ± 1 ºC for a total of 7 days (from the beginning of the
125 experiment). Thereafter, the meristems were transferred to low-light conditions (1500 lx) and after 8 days
126 to standard conditions.

127

128 *Cryopreservation of B. bituminosa clones and hybrids*

129

130 For this experiment, meristems of the genotypes CN, LB, P1, P4, P16, F1, F24, F38, and F45 were used (Table 1). After 1 day on MS03 in the dark, 27 meristems of each genotype were cultured on MS (NC). The remaining meristems (about 90 of each genotype) were transferred to polystyrene tubes with LS for 20 min. Then, the LS was replaced by ice-cooled PVS2 at 0 ºC, which was maintained for 30 min. About 30 meristems of each genotype were then transferred to RS for 15 min (NC+ PVS-30; from here onwards NC+PVS) and the rest (60) were cryopreserved (C) in liquid N_2 following the droplet procedure. Then, the C meristems were recovered in RS for 15 min. After this, the NC+PVS and C meristems were cultured in plates with MS. We executed three independent repetitions, each consisting of two Petri dishes with each Petri dish containing three NC+PVS and nine C meristems per treatment. After 7 days in darkness, the meristems were transferred to low-light conditions (1500 lx) and, 8 days after, to standard conditions. At 45 days of culture, four shoots (>5 mm) of the genotypes CN, LB, F24, F38, and P1 from cryopreserved material were transferred to tubes containing MS, whereas C and NC+PVS meristems of genotypes F1, F45, P4, and P16 were transferred to Petri dishes with fresh MS for another 45 days of culture. After this period, rooted plants grown in tubes were acclimatized in a growth chamber, in pots containing a 2:1 peat: vermiculite mixture and regeneration was assessed in the meristems transferred to fresh medium.

146 The data were subjected to factorial analysis of variance (ANOVA). The percentage data were subjected to an x^2 transformation prior to analysis. For means separation, the Duncan multiple-range test was used.

149

150 Results and Discussion

151 *Determination of PVS2 toxicity and the cryopreservation suitability of genotypes CN and P6*

152

153 In this method, incubation in a vitrification solution like PVS2 is an essential preparatory step prior to cryopreservation. The exact incubation time, however, has to be adjusted for each plant species because chemical toxicity may inhibit meristem growth. The vitrification method used in this study has already proven its efficacy for different species, resulting in high recovery rates [19, 5]. Shoot meristems are often chosen as starting explants for cryopreservation because (i) they are highly regenerable and

regenerate into a normal plant and (ii) meristematic cells contain relatively small vacuoles (with small amounts of water). As such, less water needs to be removed to prevent lethal ice crystallization, compared to non-meristematic cells [18]. The meristems were first pre-cultured for 1 day on a medium containing 0.3 M sucrose. During this period it is to be expected that tissues subjected to such a mild osmotic stress would increase their accumulation of endogenous desiccation-protecting compounds, such as abscisic acid (ABA) and/or Late Embryogenesis Abundant proteins (LEA), which can offer protection against the further dehydration stress resulting from the PVS2 incubation [3,28].

In Fig. 1, the survival (percentage of green meristems) and plant regeneration (percentage of explants giving rise to shoots >5 mm) after 90 days of culture on MS or MS BA are shown. The two accessions responded similarly to PVS2, with high values of survival (>80% on average) for non-cryopreserved and cryopreserved meristems (Fig. 1A and B). Survival after cryopreservation was not influenced by the length of the PVS2 treatment: even after 50 minutes of PVS2 treatment no decrease in survival was observed. For the C meristems, survival on MS medium supplemented with BAP was higher than on the normal MS medium; however, this difference was only significant for the 10-min PVS2 treatment with the accession CN. The duration of the PVS2 treatment had an effect on the regeneration of both the NC and C meristems. For NC meristems, of both genotypes, regeneration decreased as the PVS2 incubation lengthened (Fig. 1C and D). For cryopreserved meristems, the optimal time of incubation in PVS2 was 30 min, for both accessions and both media (Fig. 1C and D). We postulate that, after a too-short treatment (for example, 10 min), too much water remains in the cells and crystallization takes place - resulting in cell death. A too-long treatment (for example, 50 minutes) possibly results in over-dehydration and toxicity [20]. The differing effects of the duration of the PVS2 treatment on survival and regeneration are explained by the fact that individual cell integrity is needed for survival while regeneration depends on the tissue integrity of the meristematic dome.

In conclusion, an incubation of 30 min in PVS2 resulted in more than 80% regeneration in both genotypes after cryopreservation. Such percentages are promising for the establishment of a cryobank. The difference obtained between survival and regeneration shows the importance of observing regeneration instead of survival; otherwise, the wrong conclusions are drawn.

The growth aspects of the treated meristems are visualized in Fig. 2. A high amount of callus was already visible at 45 days of culture and the most prominent callus formation was observed for

187 explants cultured on MS BA, compared to MS (Fig. 2 A-B). At 90 days of culture, healthy shoots (≥1 cm)
188 were observed in meristems cultured previously on MS or MS BA medium. In P6, as well as in some
189 cultures of CN grown on MS BA, more than one shoot per culture was regenerated - indicating possible
190 adventitious (*de novo*) shoot formation. Since direct regrowth without adventitious organogenesis is
191 preferred, to reduce the chance of somaclonal variation, we selected MS for meristem outgrowth in
192 further experiments.

193

194 *Cryopreservation of B. bituminosa clones and hybrids*

195

196 To test the broad applicability of the optimized cryopreservation protocol, an experiment was
197 performed on two *B. bituminosa* clones (CN and LB) and seven *Bituminaria* intervarietal hybrids (F1,
198 F24, F38, F45, P1, P4, and P16). The genotype CN, used in the previous experiment, was included as a
199 control. Since growth ability (vigor) and PVS2 toxicity may differ among genotypes, non-cryopreserved
200 meristems that were not treated with PVS2 (NC) and those treated with PVS2 for 30 min (NC+PVS) were
201 used as controls.

202 The ANOVA analyses of the survival and regeneration revealed highly significant effects of
203 genotype, treatment, and the interaction genotype × treatment (Table 2). Although 15 days of culture is
204 too early to draw final conclusions, the fact that three genotypes (LB, F24, and P1) showed similar, high
205 survival rates of the NC+PSV and NC meristems indicates a higher tolerance/vigor of these genotypes. At
206 45 days of culture, similar survival rates were observed in the NC, NC+PVS, and C meristems of CN,
207 LB, F1, F24, F38, and P1. In F45 and P16 meristems, however, survival was greatly reduced by treatment
208 with PVS2. The best genotype was P1, with 96%, and the worst P16 and P45, with 0 and 1.8%
209 regeneration, respectively. In order to reduce PVS2 toxicity, the time of exposure to the vitrification
210 solution should be optimized [11] for such sensitive genotypes. Alternatively, the vitrification solution
211 should be changed to PVS3 (that contains only glycerol and sucrose) - which has been used successfully
212 for other species like *Brassidium* Shooting Star orchid [16] and peach palm (*Bactris gasipaes*) [6]. Also,
213 preculture of shoot-tip donor plants for 14 days in cold conditions or on media containing high sucrose
214 concentrations (for example, 0.3 M) proved to be successful for the droplet vitrification protocols applied
215 to *Byrsonima intermedia* [2] and potato [3], to increase tolerance of PVS2. With respect to regeneration

216 after cryopreservation, the average regeneration rate over all the accessions at 45 dpc was 25%.
217 Significant correlation between survival and regeneration at 45 dpc was obtained ($R^2 = 0.75$).

218 As soon as 45 days after rewarming, shoots of 3-5 cm were observed for cryopreserved meristems of
219 accession P1 (Fig. 3), which showed similar percentages of developed meristems in NC, NC+PSV, and C
220 meristems at both 15 and 45 dpc (Table 2). This genotype is a hybrid line, *B. bituminosa* var. *bituminosa*
221 (Mediterranean) x *B. bituminosa* var. *albomarginata*, selected in the breeding program for its high
222 biomass production (Table 1). Among the other genotypes selected for their high biomass production
223 (LB, F24, F45, and P4), only NC meristems of LB showed a similar growth rate at 45 dpc. Variability of
224 callus production was also observed among accessions: calli were observed in CN, F24, F38, and F45 (2-
225 5 mm diameter) as well as in P1 and LB (6-8 mm), whereas no formation of calli was observed in F1, P4,
226 or P16 at this time. These differences could be due to differing contents of endogenous growth regulators
227 in these genotypes, which may also influence meristem development.

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Shoots from cryopreserved and recovering meristems of the genotypes CN, LB, F24, F38, and P1 were transferred to tubes for rooting 30-45 days after cryopreservation and were transferred to pots for acclimatization another 45 days later (Fig. 4 A-C). Other accessions with slower regeneration rates (<13%), like F1, F45, P4, and P16, needed first to be subcultured to fresh MS medium after 90 days. After this period, the regeneration values of these four accessions increased to 63.3% (for F1), 8.9% (F45), 58.9% (P4), and 4.4% (P16) and non-pronounced callus formation was observed. Regeneration of plants from cryopreserved meristems was thus achieved in all tested genotypes at 90 dpc.

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Our results show that the droplet vitrification protocol is suitable for the cryopreservation of eight out of the 10 accessions tested. Further optimization - using cold hardening, preconditioning, and/or adapted loading and dehydration - is needed for cryobanking the genotypes with lower post - cryopreservation regeneration. Potted plants regenerated from cryopreserved material showed no morphological changes compared to the non-frozen material.

240 **Conclusions**

241 The droplet vitrification method on aluminum foil strips was successfully applied to
242 cryopreserve meristems of all tested *B. bituminosa* accessions, including two clones and eight
243 interspecific hybrids derived from crosses among the three varieties of *B. bituminosa* (*bituminosa*,

244 *crassiuscula*, and *albomarginata*). Differences in vigor, PVS2 sensitivity, tolerance of cryopreservation,
245 growth rate, and callus formation have been demonstrated among the tested genotypes. However, the
246 cryopreservation methodology employed allowed us to obtain acceptable rates of survival and
247 regeneration of normal plants from cryopreserved meristems. Hence, the method presented here can be
248 applied to develop a cryobank of *B. bituminosa* accessions.

249

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Table(s)

Table 1.

Bituminaria bituminosa and *B. bituminosa* hybrids from different regions of Spain used for the cryopreservation experiments. Each material is cloned *in vitro* from an original plant (*bitu = var. bituminosa, albo= var. albomarginata, crass=var. crassiuscula,* Med= Mediterranean origin, Can= Canary origin).

Plant material	Code	Geographical origin	Type	Origin	Characteristics
Calnegre	CN	Mediterranean region (Calnegre, Murcia	Native	*bitu*-Med	Drought tolerant, coastal, perennial, high level of furanocoumarins
Llano del Beal	LB	Mediterranean region (Llano del Beal, Murcia, Spain)	Native	*bitu*-Med	Drought tolerant, perennial, high biomass yield
OMV33E-F1-A15	F1	Canary Island (Tenerife, Spain)	Hybrid breeding line	*bitu*-Can x *albo* x *bitu*-Med	Drought tolerance, vigorous, perennial, high biomass yield
NEA11E-F24-A1	F24	Canary Island (Tenerife, Spain)	Hybrid breeding line	*albo* x *bitu*-Can x *crass*	High biomass yield
E7P-F38-A13	F38	Canary Islands (Famara, Lanzarote)	Hybrid breeding line	*albo* x *bitu*-Can	Drought tolerance, high biomass yield
E7P-F45-A3	F45	Canary Islands (Famara, Lanzarote)	Hybrid breeding line	*albo* x *bitu*-Med *x crass*	Drought and cold tolerant, high biomass yield
B3-P1-R3	P1	Mediterranean region (Llano del Beal, Murcia)	Hybrid breeding line	*bitu*-Med x *albo*	High biomass yield
B4-P4-R2	P4	Canary Islands (Las Cañadas del Teide, Tenerife)	Hybrid breeding line	*albo* x *crass*	Cold tolerant, high biomass yield
P6-A6-Bullas	P6	Mediterranean region (La Perdiz, Murcia)	Hybrid breeding line	*bitu*-Med x *albo*	Cold tolerant, high level of furanocoumarins
B4-P16-R6	P16	Canary Islands (Tenerife)	Hybrid breeding line	*bitu*-Can x *albo* x *crass*	Vigorous, perennial, high furanocoumarin content

Table(s)

Table 2

Effect of genotype x treatment, and their interaction on *in vitro* regeneration (S; survival - percentage of explants with green meristems) and shoot growth (R; regeneration- percentage of explants giving rise to shoots > 5 mm) 15 and 45 days after rewarming and culture (days post culture: dpc).

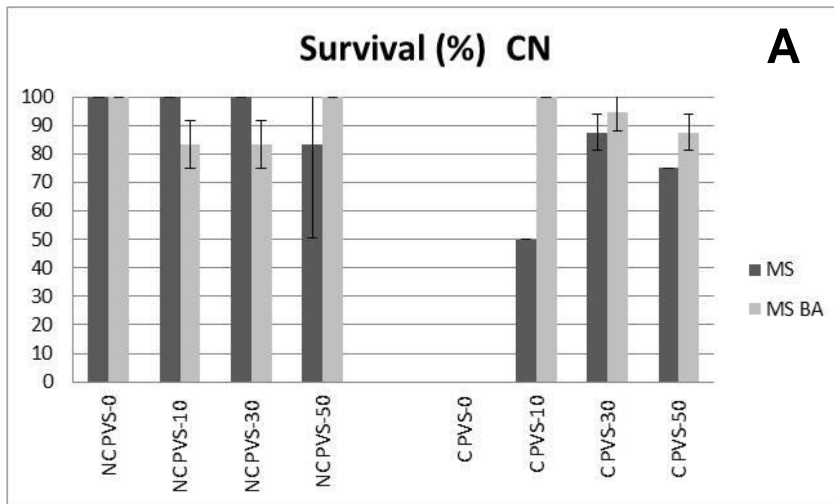
	15 dpc		45 dpc	
Genotype × Treatment Interaction	S[a]	R	S[a]	R
CN-NC	100.0 a	22.0 bcd	100.0 a	62.3 cde
CN-NC+PVS	27.5 efgh	0.0 e	94.3 ab	33.0 fgh
CN-C	29.3 efgh	1.8 e	90.2 ab	36.7 efg
LB-NC	84.7 ab	47.7 a	100.0 a	96.0 ab
LB-NC+PVS	88.7 ab	5.5 e	94.3 ab	22.0 fghi
LB-C	73.3 abcd	5.5e	86.5 ab	20.2 fghi
F1-NC	88.3 ab	25.7 bc	96.0 a	88.3 abc
F1-NC+PVS	38.7 efg	11.0 cde	83.0 ab	33.0 fgh
F1-C	31.2 efgh	9.2 de	84.7 ab	11.0 ghi
F24-NC	100.0 a	33.0 b	100.0 a	96.0 ab
F24-NC+PVS	83.2 ab	0.0 e	100.0 a	16.5 fghi
F24-C	58.7 bcde	9.2 de	86.7 ab	23.8 fghi
F38-NC	77.3 abc	11.0 cde	100.0 a	81.0 abc
F38-NC+PVS	27.5 efgh	0.0 e	66.2 abc	16.5 fghi
F38-C	25.7 efgh	3.7 e	79.0 abc	23.8 fghi
F45-NC	88.3 ab	29.3 b	100.0 a	84.7 abc
F45-NC+PVS	49.7 cdef	5.5 e	60.8 bc	11.0 ghi
F45-C	22.0 fgh	5.5 e	22.0 d	1.8 i
P1-NC	100.0 a	47.7 a	100.0 a	100.0 a
P1-NC+PVS	94.3 a	49.5 a	94.3 ab	86.8 abc
P1-C	92.2 ab	53.2 a	98.0 a	96.0 ab
P4-NC	77.0 abc	3.7 e	85.0 ab	70.0 bcd
P4-NC+PVS	44.2 def	0.0 e	60.7 bc	23.8 fghi
P4-C	28.5 efgh	0.0 e	47.7 cd	12.8 ghi
P16-NC	80.7 abc	0.0 e	96.0 a	44.0 def
P16-NC+PVS	5.5 gh	0.0 e	49.8 cd	5.5 hi
P16-C	1.8 h	0.0 e	22.2 d	0.0 i
ANOVA[b]				
Genotype	***	***	***	***
Treatment	***	***	***	***
Genotype × Treatment	*	**	*	**

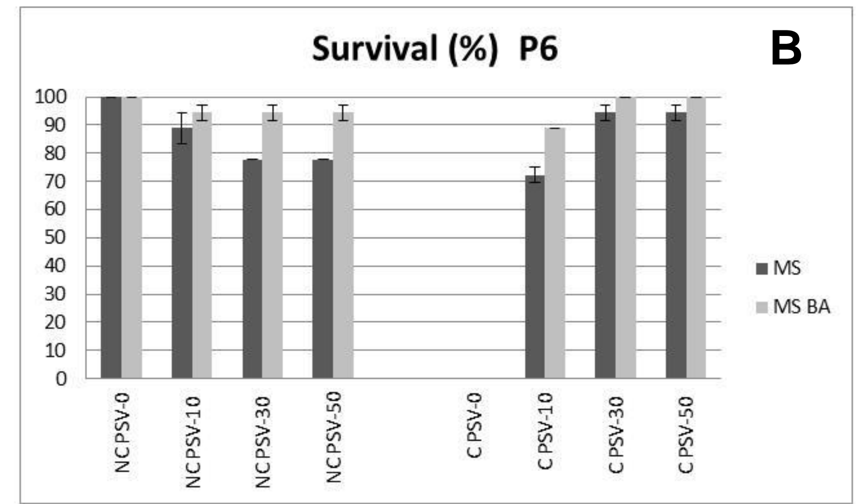
[a]For each of the genotype, treatment, and genotype × treatment interaction factors, mean values within a column separated by different letters are significantly different (P<0.05) according to Duncan's multiple range test.

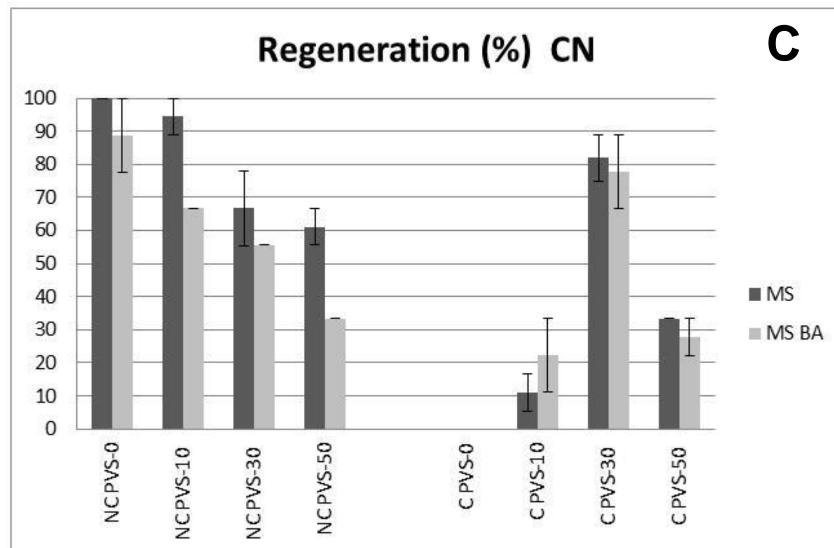
[b]***, **, *, and ns indicate significant at P<0.001, P<0.01, P<0.05, and non-significant, respectively.

C: cryopreserved, NC: non-cryopreserved, NC+PSV (non-cryopreserved and incubated for 30 min with PSV).

Figure(s)







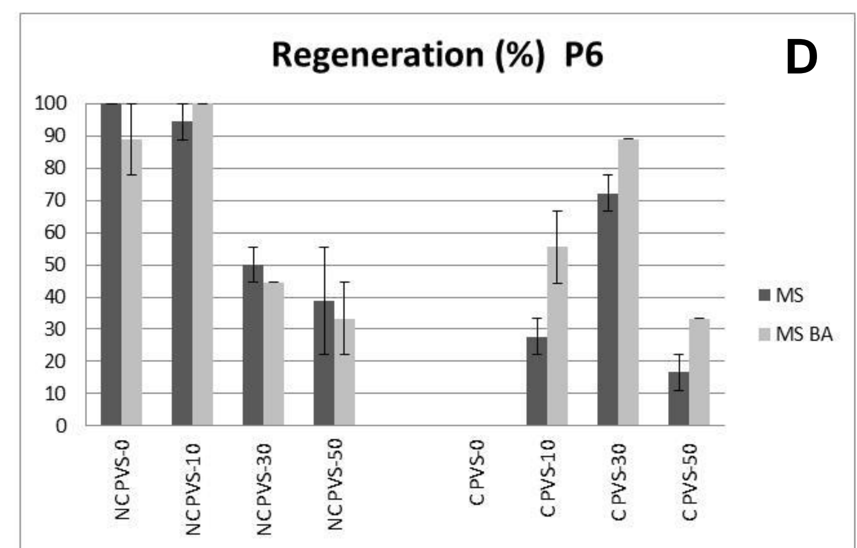


Fig 1.

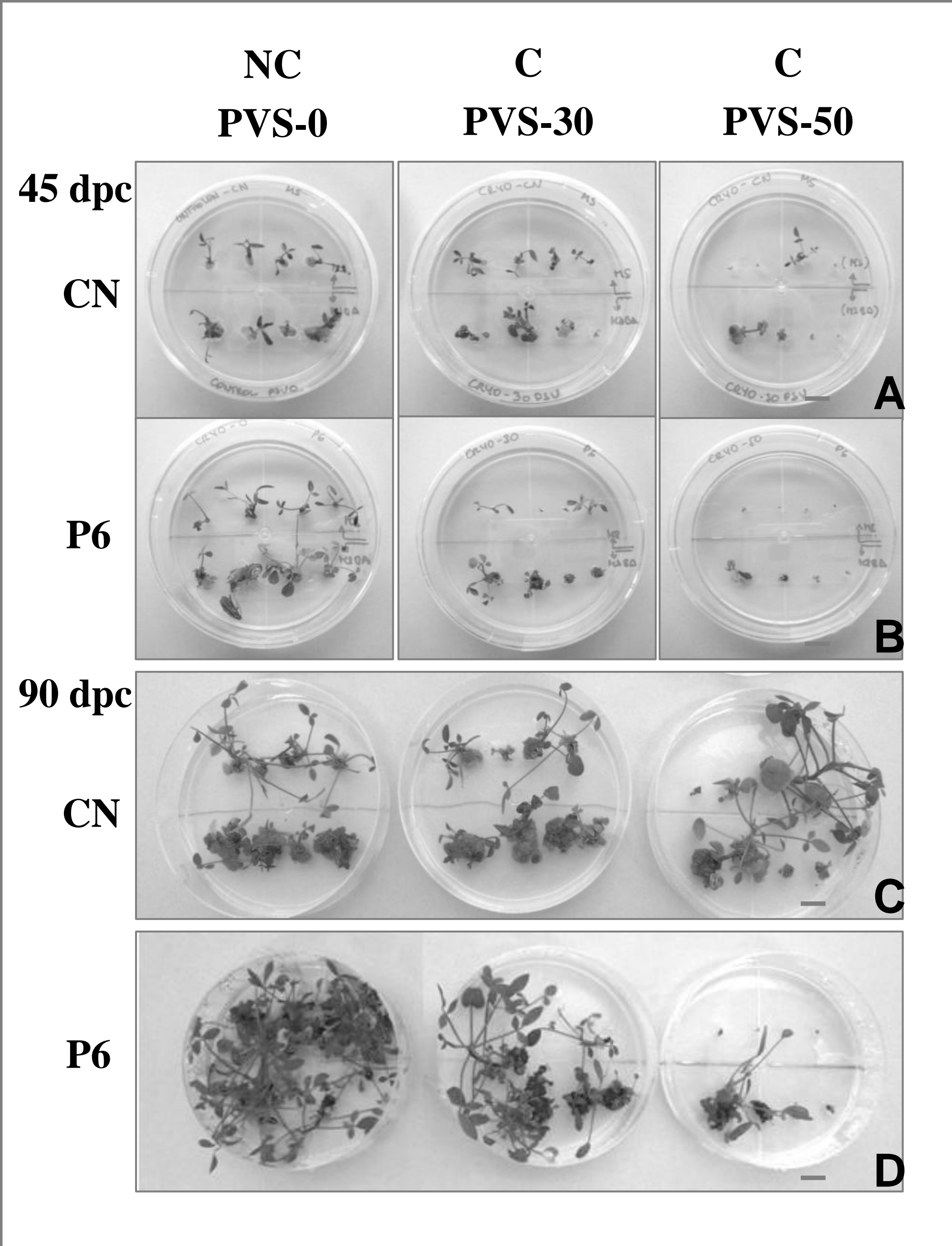


Fig. 2.

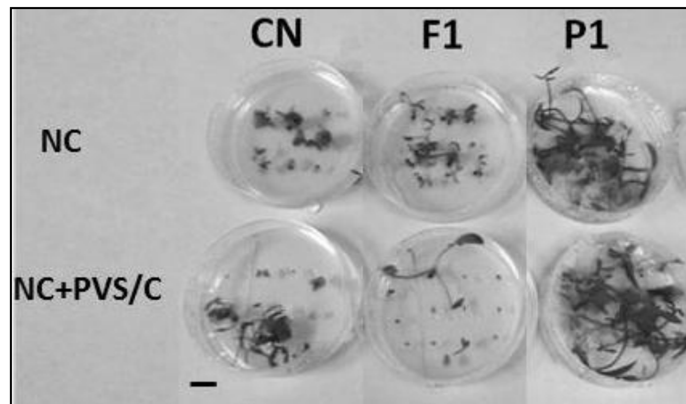


Fig. 3.

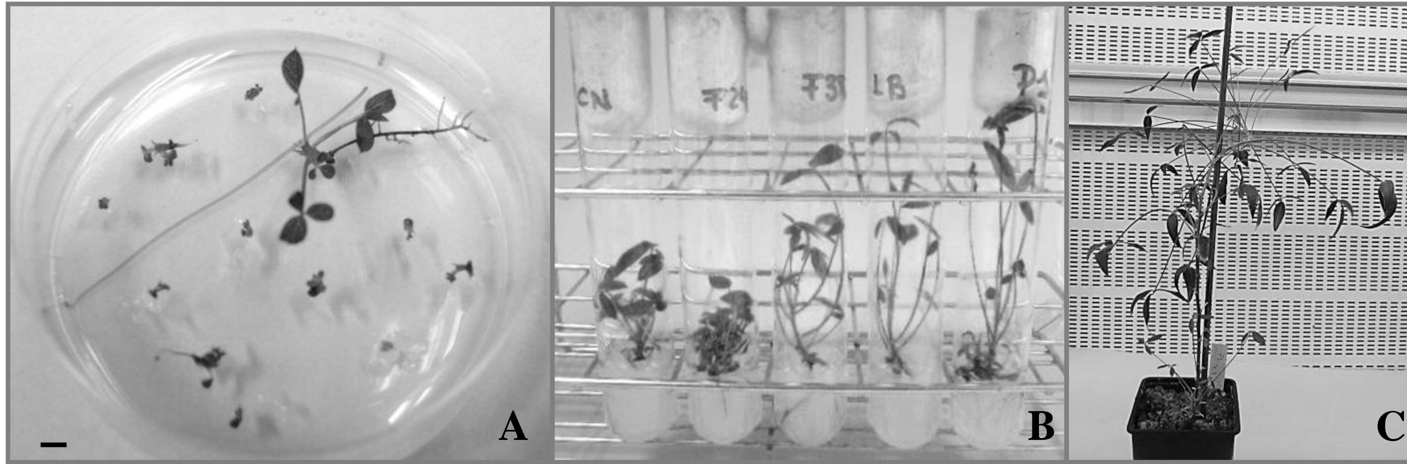


Fig. 4.

Figure(s)

Fig. 1. Survival (A and B) and plant regeneration (C and D) of *Bituminaria bituminosa* (genotypes CN and P6) meristems after 90 days of culture on MS medium or MS medium supplemented with BAP (MS BA). PVS-0, PVS-10, PVS-30, and PVS-50: incubation of explants for 0, 10, 30, and 50 min, respectively. For each PVS treatment, four Petri dishes with 8-10 meristems of each genotype were treated. Half of the samples per PVS treatment were immersed in liquid nitrogen before being placed in RS (Cryopreserved; C) and half were placed directly in RS (Non-cryopreserved; NC).

Fig. 2. Non-cryopreserved (NC) and cryopreserved (C) meristems of genotypes CN and P6, incubated with PVS2 solution for 0 (PVS-0), 30 (PVS-30), or 50 (PVS-50) min and cultured on MS or MS BA for 45 (A and B) or 90 (C and D) days and then transferred to tubes with MS for 45 days. The upper half of each plate represents meristems cultured on MS and the lower half those cultured on MS BA. Scale bar: 5 mm.

Fig. 3. Meristems (0.3-0.5 mm) of genotypes CN, F1, and P1 after 45 days of culture on MS medium. The upper photos show nine meristems without PSV2 treatment and cryopreservation (NC) in each plate. The lower photos show, in each plate, three meristems treated with PSV2 but not cryopreserved (NC+PSV, left side of each dish) and nine cryopreserved meristems after PSV2 treatment for 30 minutes (C, right side of each dish).

Fig. 4. **A.** Three non-cryopreserved meristems (upper left side) and nine cryopreserved meristems (lower right side) of genotype LB (both treated with PVS2 for 30 min), after 45 days of culture on medium MS. **B.** Growth of cryopreserved meristems of genotypes CN, F24, F38, LB, and P1, from left to right, respectively, 45 days after their transfer to tubes containing MB. **C.** Plant of genotype P1 (from a cryopreserved meristem) acclimatized in a growth chamber. Scale bar in A: 5 mm.