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Seed cryopreservation of *Fraxinus angustifolia* Vahl

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***Fraxinus angustifolia* is used for afforestation and for production of both valuable timber and *manna*, a substance with pharmacological applications. The aim of this research was to establish the optimized condition for cryopreservation of *F. angustifolia* seeds, in order to protect the germplasm of this species. Germination percentage and mean time of germination of non-treated seeds (control) and liquid nitrogen-treated seeds were established in water or in 10^{-6} M gibberellic acid (GA_3). The seeds could be cryopreserved with 3% of moisture content (MC) and germinated easily in water (70.0 ± 5.0%), while seeds cryopreserved with 6% MC showed a physiological dormancy. This state could be efficiently removed by addition of GA_3, which increased germination to 76.0 ± 2.4%. Liquid nitrogen did not impair embryo viability, allowing a successful cryopreservation of seeds. The results are particularly useful for both germplasm conservation programs and in narrow leaved ash nurseries.**

Key words: *Fraxinus angustifolia*, germination, germplasm conservation, seed cryopreservation.

INTRODUCTION

Fraxinus angustifolia Vahl (narrow leaved ash) is a woody species used in afforestation and production of valuable timber. In Sicily (Italy), this species is cultivated together with *Fraxinus ornus* L. to produce the “manna” (Ilardi and Raimondo, 1999), a solidified phloem sap collected from artificial incisions on the trunks. Manna has many pharmacological applications (Schicchi et al., 2006). Ash cultivations for manna production are located exclusively in small areas of Castelbuono and Pollina (Palermo, Sicily) and are gradually disappearing since this activity is mainly carried out by old farmers. Some ash individuals are very ancient and so big to be considered monumental. In the district of Puntaloro (Petralia Sottana, Palermo), there is one of the oldest specimens of *F. angustifolia* in Italy. This individual is 400 years old, 28 m high and has a trunk of 7.5 m round. Pests, diseases and environmental adversities negatively and severely influence the growth of this monumental ash, making necessary a specific preservation strategy by *ex situ* conservation methods and techniques which allow its re-introduction in nature.

The storage of biological material in liquid nitrogen (Brearley et al., 1995) is the best method currently used to preserve and store genetic resources for long-term *ex situ* conservation. It has been suggested that the extremely low temperature of liquid nitrogen (-196°C) arrest cell metabolism (Engelmann, 2004), although some studies have shown a residual activity of some ageing reactions (Walters et al., 2004). Ash seeds are classified as orthodox (Bonner, 2008). The seeds with

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Abbreviations: Cryo, Cryopreserved seeds; **GA_3,** gibberellic acid; **LN,** liquid nitrogen; **MC,** moisture content; **MTG,** mean time of germination; **NT,** non-treated seeds; **TTC,** 2,3,5-triphenyl tetrazolium chloride.

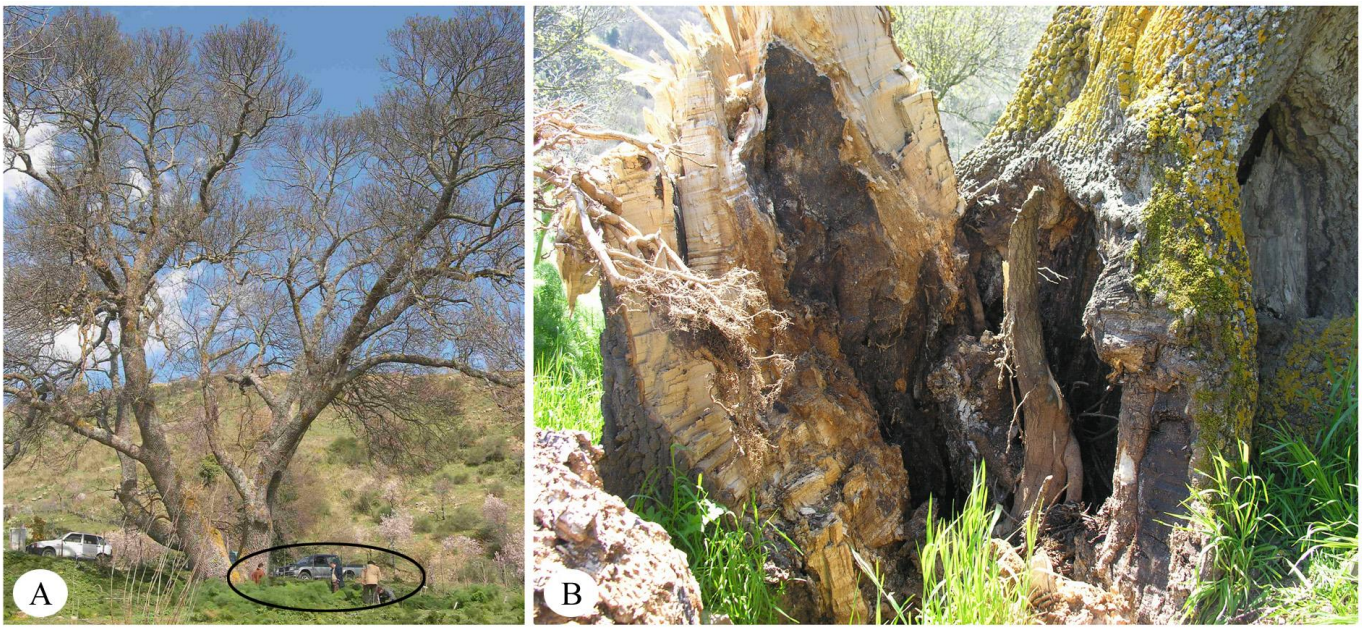

Figure 1. Monumental specimen: 400-year-old *F. angustifolia* (A) and the detail of a breaking point of the trunk (B) due to diseases and environmental adversities. In the circle, the men's size is reference.

orthodox behaviour (Roberts, 1973) represent a good biological type suitable for cryopreservation because they can tolerate freezing without using cryoprotectors (Chetverikova, 2008) as long as they are sufficiently dry (Popov et al., 2006) since the seed moisture content is a critical factor determining the viability and longevity of the seed.

Seeds of *Fraxinus excelsior* L. can be successfully stored in liquid nitrogen (Chmielarz, 2009), but no information about seed cryopreservation of *F. angustifolia* is available to date. The seeds of the genus *Fraxinus* undergo different types of dormancy, according to the specific characteristics of the embryo: morphological dormancy or physiological dormancy. The first occurs when the seed is relatively small though morphologically complete, the latter occurs when the seed is still dormant at seed dispersal time; moreover, a delay in germination is often caused by the mechanical resistance offered by the enveloping tissues, that is, endosperm, suberized layer and seed coat (Preece et al., 1995; Steinbauer, 1937). Since the degree of embryo development and the dormancy status of the seed vary considerably among the different species of ash, it is important to assess the most suitable cryopreservation methods for narrow leaved ash seeds.

The aim of this research was to verify the viability of *F. angustifolia* seeds after storage in liquid nitrogen in order to preserve its invaluable germplasm.

MATERIALS AND METHODS

Mature brown samaras of a monumental tree of *F. angustifolia*, located in Puntaloro (Petralia Sottana), Palermo-Italy (Figure 1), were collected at the end of July (early stage of maturation) and at the middle of November (final stage of maturation) (the blossom occurred in December-January). Seeds were extracted from the fruits and the percentages of seed presence/absence, number of seeds per samara and aborted seeds were evaluated. Seeds were considered aborted when their width was lower than 15% of the mean width.

The moisture content (MC) of the seeds was measured in five independent replicas of 10 seeds each, by drying at 103 ± 2°C for 18 h, repeating the treatment until seeds had reached constant dry masses. The MC was expressed as percentage of fresh weight. In order to process all seeds at once, seeds harvested in July were stored at 4°C with moisture content (MC) of 8 to 10% until November. Before starting the experiments, the MC was decreased to 6% in the seeds harvested in July (group 1) and to 3 (group 2) and 6% (group 3) in the seeds harvested in November. In the latter lot, a significantly higher seed number allowed assessment of the influence of two different MC percentages on viability of cryopreserved seeds. The dehydration was performed in sealed jars containing silica gel, previously oven-dried at 130°C for 24 h.

The seeds to be cryopreserved (Cryo) were placed in cryovials, plunged directly into liquid nitrogen (LN) and stored for 10 days. After freezing in LN, the cryovials were thawed at 40°C in a water bath for 15 min. Non-treated (NT) seeds were used as control. Before starting the germination experiments, NT or cryopreserved seeds from each group, were sterilized by immersion for 1 min in ethanol (70% v/v in water), 20 min in 30% (v/v in water) commercially-available bleach containing few drops of Tween 20, followed by three rinses with sterile distilled water. The seeds were left imbibed for 24 h in a laminar flow hood. For germination experiments, seeds were incubated in 9-cm diameter Petri dishes containing four layers of filter paper (Whatman n°1) and imbibed with 10 ml distilled water (control) or 10^{-6} M GA_3 at 20°C and 16/8 h of light/darkness and light intensity of 50 µmol/m^2/s provided by Osram cool-white 18 W fluorescent lamps. Each experiment was performed with 10 seeds and repeated 20 times. To estimate the highest value of germination, germination percentage was recorded

Table 1. Germination percentage after 30 and 60 incubation days, mean time of germination (MTG) after 30 days of incubation, root and hypocotyl lengths of seedlings developed from 30 days incubated seeds, belonging to the three groups.

Group		Germination		MTG	Length	
	Incubation day	30	60	30	Root	Hypocotyl
	Treatment					
1	NT H_20	26.0±4.0^{a}	26.0±4.0^{a}	26.0±4.0^{a}	27.5±2.5^{a}	9.0±1.0^{a}
	NT GA_3	15.7±1.4^{b}	15.7±1.4^{b}	15.7±1.4^{b}	33.4±3.8^{ab}	21.1±2.3^{bd}
	Cryo H_20	26.7±3.3^{a}	26.7±3.3^{a}	26.7±3.3^{a}	20.6±13.7^{a}	9.6±4.8^{a}
	Cryo GA_3	18.4±0.9^{ab}	18.4±0.9^{ab}	18.4±0.9^{ab}	32.5±3.6^{ab}	17.1±1.9^{abd}
2	NT H_20	9.8±0.3^{b}	9.8±0.3^{b}	9.8±0.3^{b}	51.5±7.2^{b}	17.0±1.3^{abd}
	NT GA_3	10.6±1.4^{b}	10.6±1.4^{b}	10.6±1.4^{b}	38.5±4.4^{ab}	23.6±1.9^{bc}
	Cryo H_20	9.9±2.3^{b}	9.9±2.3^{b}	9.9±2.3^{b}	46.0±6.9^{ab}	17.1±2.8^{abd}
	Cryo GA_3	8.6±0.6^{b}	8.6±0.6^{b}	8.6±0.6^{b}	33.7±2.6^{ab}	34.4±1.3^{c}
3	NT H_20	15.3±1.2^{b}	15.3±1.2^{b}	15.3±1.2^{b}	50.8±8.2^{b}	11.6±1.6^{a}
	NT GA_3	9.7±1.5^{b}	9.7±1.5^{b}	9.7±1.5^{b}	34.6±2.8^{ab}	33.7±2.0^{c}
	Cryo H_20	13.0±2.1^{b}	13.0±2.1^{b}	13.0±2.1^{b}	42.2±5.8^{ab}	12.4±1.4^{a}
	Cryo GA_3	9.5±0.8^{b}	9.5±0.8^{b}	9.5±0.8^{b}	29.2±2.7^{a}	26.9±1.7^{cd}

Mean ± standard error. Means followed by the same letter are not significantly different at $p<0.05$, according to the Tukey's test. 1, Seeds harvested in July and incubated with 6% moisture content (MC); 2, seeds harvested in November and incubated with 3% MC; 3, seeds harvested in November and incubated with 6% MC of seeds not treated (NT) or treated in liquid nitrogen (Cryo) incubated in water (H_20) or in 10^{-6} M gibberellic acid (GA_3).

weekly for 30 days and after 60 days.

Mean time of germination (MTG) was calculated after 30 days and was determined according to the formula: $[\Sigma n_i d_i/n]$, where n_i is the number of seeds that germinated at day d_i; d_i is the considered day and n is the total number of seeds that germinated in the incubation period. Seeds with radicle length of 2 mm were considered as germinated. After 30 days of incubation, the lengths of roots and hypocotyls in developed seedlings were measured.

After 60 days, non-germinated seeds were stained in 1% 2,3,5-triphenyl tetrazolium chloride (TTC) dissolved in tap water (pH 7.0) to test their viability (Patil and Dadlani, 1993). Seeds were cut laterally along the longitudinal axes and completely covered with TTC solution. Samples were incubated in the dark at 25 ± 2°C for 20 h, rinsed twice with water and immediately observed under the stereo microscope. Intermediate or white-stained seeds were considered as non-viable, while exclusively red embryos were considered as vital.

To verify the vigour of cryopreserved seeds, 30 to 60 days-old seedlings obtained from cryopreserved seed were transferred in Jiffy® into magenta and incubated at 25°C and 16/8 h of light / darkness; when the radical apparatus became visible in the Jiffy®, the seedlings were potted, closed in transparent plastic bags and placed in the greenhouse. The plastic bags were perforated gradually to bring the seedlings in full air.

Statistical analyses were performed with Sigmastat 3.5 software. To evaluate the significance of the differences between the mean values, analysis of variance (ANOVA) and Tukey test for pair-wise comparison at $p<0.05$ were applied.

RESULTS

Among the seeds collected in July (group 1), 87.7% of samaras contained one normal seed, 4.5% two seeds, 2.4% aborted seeds and 5.4% were empty. Among the seeds collected in November (groups 2 and 3), the samaras containing one normal seed were 76.6%, 7.5% contained two seeds, 0.4% three seeds, 7.5% aborted seeds and 8.0% of the samaras were empty.

The MC of seeds collected in July (6.4 ± 0.1) was slightly but significantly ($p<0.001$) reduced compared to the one of those collected in November (8.0 ± 0.1).

After 30 days of incubation, the germination percentage (Table 1) of group 1 was very low in water, 4.0 ± 1.0% for NT seeds and 6.0 ± 1.0% for the Cryo seeds. The GA_3 treatment increased the germination percentage to 76.0 ± 2.4% for NT and 82.0 ± 2.0% for the Cryo, which reached the highest percentage of germination. Furthermore, a significant difference between incubation in water or GA_3 was registered ($p<0.001$) while no significant effect of the cryopreservation treatment could be observed. GA_3 speed up the germination process, decreasing the MTG by 10.3 days ($p<0.05$) in NT seeds and 8.3 days in Cryo seeds. Group 2 showed a homogeneous germination percentage ranging from 64.0 ± 2.0% (NT in water) to 76.0 ± 4.0 (NT in GA_3), with an intermediary value of 70.0 ± 5.0% (Cryo seeds in water and GA_3). No significant difference within these groups was observed. Accordingly, also, the MTG among groups was not significantly different, ranging from 8.6 to 10.6 days. In group 3, the highest germination percentage was recorded in GA_3-treated seeds with values of 74.0 ± 2.4 and 76.0 ± 5.0, respectively, in NT and Cryo. The plant regulator showed a significant effect ($p<0.001$) on germination probability over the water incubation, which led to germination percentages ranging from 44.0 ± 2.4 (NT) to 56.0 ± 4.0 (Cryo). The MTG was lower in seeds incubated in GA_3 (9.7 and 9.5

days, respectively, in NT and Cryo seeds), while the MTG in water increased by 5.6 days for NT seeds and 3.5 for Cryo seeds. After 60 days of incubation, the germination percentage slightly increased in all the these except for NT GA_3 and Cryo H_2O of Group 2, and Cryo GA_3 of Group 3 (Table 1).

The root length (Table 1) ranged from 20.6 ± 13.7 to 51.5 ± 7.2 mm and was longer in groups 2 and 3 than group 1, but no significant difference could be observed. The hypocotyl length (Table 1) ranged from 9 ± 1 to 34.4 ± 1.3 mm. In this case, the seeds harvested in November and incubated in GA_3 showed a significantly longer hypocotyl as compared to the other treatments. The seedlings belonging to the three groups did not exhibit any morphological difference among the treatments as shown in Figure 2A to D; however, during the development stage, the plantlets originated from seeds incubated in GA_3 showed a longer epicotyl (Figure 2F) as compared to those incubated in water (Figure 2E).

After 60 days of incubation, seeds were opened to quantify necrotic embryos (Figure 3A). Necrotic embryos were respectively 7.2 ± 1.3% in group 1, 6.6 ± 0.5% in group 2 and 10.5 ± 1.1% in group 3. Especially in group 1, NT and Cryo seeds were necrotic, respectively, for 8.0 ± 2.3 and 5.0 ± 1.0%, in the group 2, NT and Cryo seeds were necrotic, respectively, for 6.6 ± 0.4 and 6.6 ± 1.0% and in the group 3, NT and Cryo seeds were necrotic, respectively, for 11.0 ± 2.0 and 8.0 ± 1.3%. Overall, the cryogenic treatment did not have significant effect on the necrotic status of the embryos.

As a result of the TTC test (Figure 3B), non-viable embryos were quantified as 2.0 ± 0.4% and 4.0 ± 1.0%, respectively, in NT and Cryo seeds of Group 1, 13.0 ± 2.1 and 8.3 ± 0.8% in NT and Cryo seeds of group 2 and 6.0 ± 1.0% and 4.0 ± 1.0% in NT and Cryo seeds belonging to group 3 (no statistically significant differences identified). Collectively, these results confirm that the drastic drop of temperature induced by LN does not negatively affect the viability of the embryos.

The acclimatization occurred successfully and both plants that originated from Cryo seeds incubated in water or GA_3 developed similarly (Figure 2G and H). It should be noted that despite the fact that leaves of seedlings germinated from GA_3-incubated seeds appeared scrubbier when compared to the water control seeds, at later stages of growth, plants were morphologically undistinguishable (Figure 2I).

DISCUSSION

The amount of seeds per samara is an important parameter to be considered in order to collect the appropriate amount of fruits for gene bank preservation purposes. The samaras collected in these study contained seeds in percentages ranging from 93 to 97%. Seed of *F. angustifolia* are typically characterized by a shallow degree of dormancy, as proposed by Piotto and Piccini (1998), which still observed both morphological and physiological dormancy; on the other hand, Raquin et al. (2002) considered the dormancy as an effect of either tegument or endosperm inhibition.

In our experiments, seeds harvested at dispersal time appeared complete and homogenous in size, without any observable morphological dormancy. Any mechanical resistance opposed by the enveloping tissues of the embryo to germination could also be excluded. Seeds collected in July, at early stage of maturation, exhibited exclusively a physiological dormancy which could be broken by gibberellin addition. The seeds collected in November, during the final stage of maturation, after desiccation to 3% MC did not show any dormancy. In contrast, seeds collected also in November but desiccated to 6% MC underwent physiological dormancy, as indicated by the significantly lower germination percentage in water (44.0 ± 2.4), which could be reverted by the addition of GA_3 up to 74.0 ± 5.0.

The physiological dormancy was significantly stronger in seeds collected in July with respect to those collected in November, suggesting a loss of dormancy during seed maturation. Furthermore, seed MC interfered with the germination process or the dormancy break and we suggest a dormancy-inhibitory effect of 3% MC. The lack or shallow dormancy observed in *F. angustifolia* is in agreement with the studies by Piotto and Piccini (1998) for two reasons. First, the germination occurred easily without pre-treatment when seeds were sowed in autumn. Second, Piotto and Piccini (1998) observed in Italy a trend of dormancy of *F. angustifolia* seeds which decreased with decreasing latitudes. Accordingly, experiments were conducted at even lower latitudes (Sicily) thereby explaining the decreased dormancy level observed. The Sicilian *F. angustifolia* does not need any stratification pre-treatment, as reported by other authors (Piotto and Piccini, 1998; Steinbauer, 1937). A similar behaviour, that is, lack or low dormancy and no stratification requirement, is supposed for the seeds of *F. angustifolia* at same latitude or similar ecological conditions.

The constant temperature of 20°C was optimal for the germination, in contrast with Piotto (1994) that found this temperature to inhibit germination and furthermore induced secondary dormancy.

To our knowledge, ash cryopreservation experiments were conducted on dormant buds of *F. pennsylvanica* Marshall, *F. mandshurica* Rupr. and *F. chinensis* Roxb. (Volk et al., 2009) and on intact seeds (Chmielarz, 2009), zygotic embryos (Brearley et al., 1995), embryogenic callus (Ozudogru et al., 2010) and shoot tips (Schoenweiss et al., 2005) of *F. excelsior.* It is not reasonable to suppose a similar behaviour between *F. angustifolia* and *F. excelsior* seeds after cryopreservation treatment. In fact the development degree of the embryos is very different between the two species, the dormancy degree of *F.* excelsior is greatly stronger as compared to *F. anfustifolia* (Piotto and Piccini, 1998; Raquin et al., 2002).

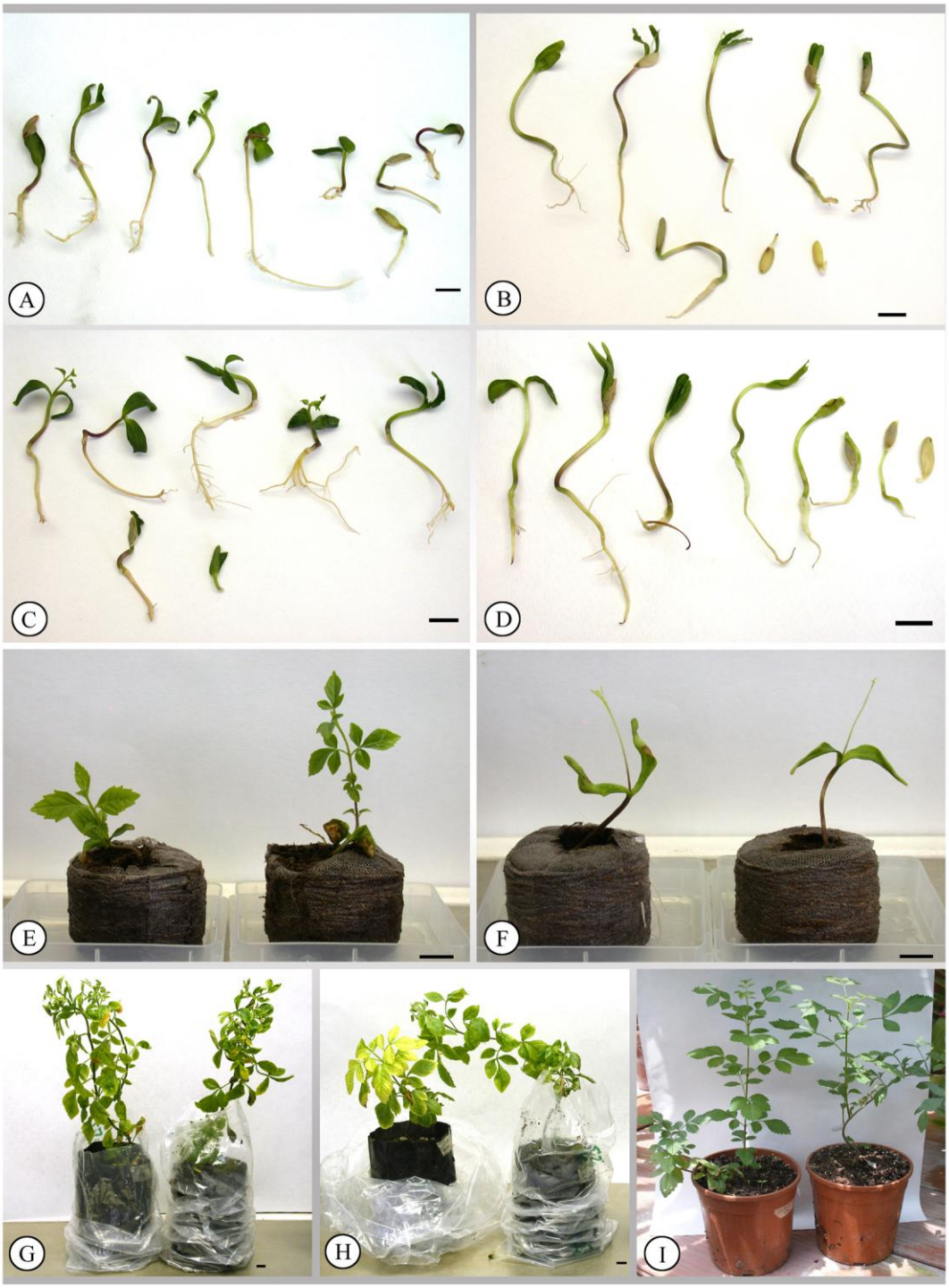

Figure 2. Developed seedlings of not treated seeds incubated in water (A), in GA_3 (B) and of cryopreserved seeds incubated in water (C) and in GA_3 (D) for 30 days, seedlings of about 45-day-old transferred to Jiffy® substrate and developed from cryopreserved seeds incubated in water (E) and in GA_3 (F), four-month-old potted plants developed from cryopreserved seeds incubated in water (G) and in GA_3 (H) and potted plants of seven-month-old (I). Bar 1 cm.

The results demonstrate the possibility to cryopreserve successfully the seeds of *F. angustifolia*. There was no significant effect of the cryopreservation treatment; in fact, both germination percentages and viability of embryos, revealed by TTC test, were statistically alike between the not treated and the treated seeds. As occurred

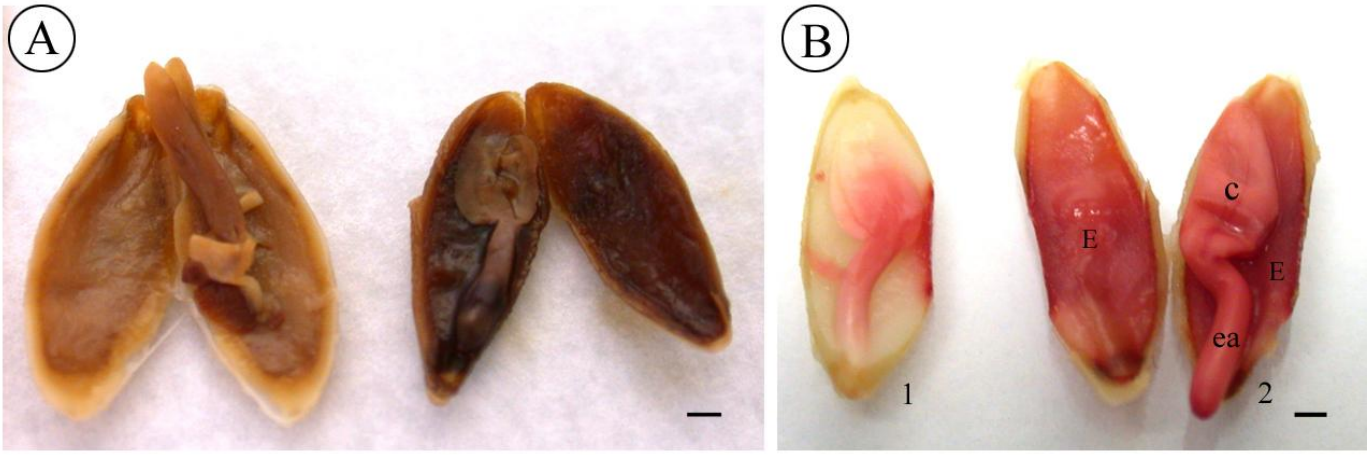

Figure 3. Necrotic embryos (A) and seeds stained with 1% TTC solution (B). 1, not viable; 2, viable embryos; c =cotyledons, E = endosperm; ea = embryo axis. Bar 1 mm.

in not treated seeds, also in cryopreserved seeds, gibberellin addition had a significant effect on germination of seeds collected in July and in November desiccated to 6% MC, while there was no significant effect of gibberellin only in cryopreserved seeds collected in November and desiccated to 3% MC. The use of full mature seeds cryopreserved with 3% MC would avoid the addition of gibberellin, that at the beginning of the plant growth, had a negative effect on epicotyl development (longer than controls) and on the leaves that were scrubby. The seeds collected in July were able to be cryopreserved with 6% MC, but to induce germination, the addition of gibberellin is essential. The possibility to cryopreserve these seeds could eliminate the risk of losing the samaras due to early dissemination.

Roots and hypocotyls length were normal without any significant difference among all the thesis of treated and not treated seeds. To cryopreserve, the safe range of 3 to 6% MC in *F. angustifolia* seeds is in agreement with the results of Pritchard and Nadarajan (2008) that stated that MC lower or equal to 8% tend not to affect seed survival following cryopreservation, which is in contrast with *F. excelsior* seeds, in which MC inferior to 7% had a negative effect on the germination of cryopreserved seeds (Chmielarz, 2009).

In *F. angustifolia*, buds/shoots were successfully obtained by organogenesis (Tonon et al., 2001b) and somatic embryos by embryogenesis (Tonon et al., 2001a), so that these explants could be used in other experiments of cryopreservation if it is useful to maintain clonal lines. In germplasm conservation programs, it is important to preserve the genetic diversity and therefore to conserve the seeds which is important to ensure the biodiversity within the species.

In conclusion, the cryopreservation did not damage the seeds of *F. angustifolia.* The seeds can be harvested during the early stage of maturation and cryopreserved with 6% MC or harvested during the final stage of maturation with 3% MC. In the first case, the risk of losing the seeds because of early dissemination is avoided, but the addition of gibberellic acid is required to obtain a germination percentage >80%. In the latter case, the germination is 70% and the addition of the growth regulator is not necessary. The seedlings grew regularly and developed into plants after potting and acclimatization. This protocol is very useful for the preservation of monumental specimens of *F. angustifolia* and finds applications in the germplasm conservation of this species.

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