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Effects of pretreatments on *Corema album* (L.) D. Don (subsp. *album*) seeds' germination

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Abstract. *Corema album* (Ericaceae), "Camarinhas", endemic of the Atlantic dunes of the Iberian Peninsula has the possibility to become a new niche berry crop. Considering the agronomic and market possibilities for this species, the initial research step is the development of a seed germination protocol. We characterized *C. album* populations along its biogeographical area. The results show that different seeds characteristics differ significantly between sites. We also tested the effect of pretreatments on the germination of *C. album* seeds (subsp. *album*) from Duna de Quiaios in 2011, from Comporta in 2011 and Aldeia do Meco in 2011 and in 2012. The results show that there is site-to-site variation in the same year and year-to-year variation in a particular site in percentage of germinated seeds. Acid scarification (30, 60 and 120 minutes) followed by 1000 ppm of gibberellic acid was the most effective pretreatment in breaking dormancy of *C. album* and this pretreatment promote the best germination from seeds collected from Aldeia do Meco 2011 (30.3%) after 175 days. Our results suggest that the seeds had physiological dormancy. Considering the 2800 seeds collected: 54.5% ruptured the seed coat and 7.7% germinated.

Keywords: *Corema album*, seed dormancy, seed germination, new berry crop, pretreatments

1. Introduction

Corema album (L.) D. Don (Ericaceae) is a dioecious shrub which rarely exceeds 1 m in height, endemic of the Atlantic dunes of the Iberian Peninsula, spread along the coast from Finisterre in the NW of Galicia to Gibraltar [2, 13, 25]. However, 1-4% of male plants from the southwest present some hermaphrodite inflorescences [24, 30]. The growing period takes place from February to July, reaching its maximum between April and June, while flowering occurs from February to April, with fruits ripening from June to September [1, 25, 26]. Female plants produce edible white or pink-white berry-like drupes (5-8 mm diameter) in the middle of the branch as the terminal bud continues to grow, generally with three large pyrenes (ranging from 2 to 9) [1, 18, 25]. The high proportion of the seeds weight (54.97% of dry weight) [22], also explains that these fruits have lower edible part than other berries. The pyrenes (seeds) have a thick woody endocarp [9]. Berries are considered to be mildly acidic with a lemony flavor, which have a sugary and water-rich pulp (6.8 ± 0 °Brix and 83.41%, respectively) [22, 25, 26] and these fruits are a potentially important source of nutrients and phytochemicals (phenolic acids and flavonols) [19, 26]. The total phenolic content are 1214.4 ± 122.7 mg GAE/kg FW or 7318.6 ± 739.6 mg GAE/kg DW [22] and fruits contains high amounts of caffeic acid derivatives (39.42 ± 2.15 mg/100 g DW) [26] and have very low amounts of anthocyanins [22]. Based on this, *C. album* fruit has the possibility to become a new niche berry crop.

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Considering the agronomic and market possibilities for this species, initial breeding would be by mass clonal selection on plants grown from seed collected from populations in their native habitat. This populations need to be characterized and plants selected for large fruits, good fruit quality and small seeds [25]. So, the initial research step is the development of a seed germination protocol.

Seed germination refers to the physiological process including a series of events that begins with water uptake by the quiescent dry seed (imbibition) and ends with the elongation of the embryonic axis, usually the radicle, through the structures surrounding it [6, 7]. The visible sign that germination is complete is usually the penetration of the structures surrounding the embryo by the radicle, the result is often called visible germination [7]. A non-dormant seed has the capacity to germinate over the widest range of normal physical environmental factors possible for the genotype (and considering maternal effects) [4, 17]. If freshly matured, viable seeds fail to germinate at any of several combinations of environmental conditions, they are dormant [4]. So, a dormant seed is one that does not have the capacity to germinate in a specified period of time under any combination of normal physical environmental factors that otherwise is favourable for its germination, i.e. after the seed becomes non-dormant [17]. Five general types of dormancy are known to occur in seeds at maturity: physiological, physical, combinational, morphological, and morphophysiological [3]. These are distinguished on the bases of (1) permeability or impermeability of the seed (or fruit) coat to water, (2) whether the embryo is fully developed or underdeveloped at seed maturity, and (3) whether the embryo is physiologically dormant or non-dormant [3]. These five types are roughly based on the pretreatments required to break dormancy and response of the seed to specific conditions.

Previous studies have reported that each frugivore species plays an essential role in seed dispersal and germination, and final seedling recruitment of the species *C. album* [8–12, 14, 15, 21, 24]. So, natural regeneration in *C. album* appears to be associated with animal dispersers. Frugivorous animals, attracted by the nutritious pulps, consume the fruits and acting as dispersal agents of *C. album* seeds [10, 12]. Seed dispersers are an important link between the adult and seedling stages and can affect germination by removing the fruit pulp, which may contain germination inhibitors [10]. Moreover, mechanical and chemical scarification of the seed coat can enhance seed germination by increasing the permeability to water and gases [9]. *C. album* seeds are dispersed mainly by gulls, blackbirds, foxes and rabbits, and seed germination occurs after a dormancy period of at least 1–4 years [9].

Once it is documented that germination and seedling survival of the species *C. album* are difficult under controlled conditions, the specific objectives of this study were: (1) Characterized *C. album* populations along its biogeographical area and (2) Tested the effect of pretreatments on the germination of *C. album* seeds (subsp. *album*), under controlled conditions of temperature, relative humidity and photoperiod.

2. Materials and methods

2.1. Plant materials

White, soft fruits of *C. album* were collected in August 2011 from 7 locations in Portugal (Table 1) [25]. Additionally, fruits from 18 genotypes were collected in November 2012 from Aldeia do Meco (38° 28′ 07″N/9° 11′ 38″W) (Fig. 1).

Table 1

The location, latitude and longitude of seven sites of *Corema album* D. Don in Western Portugal collected in August 2011

Location	Latitude/Longitude
S. André	38°07′11″N/8°47′40″W
Pego	38°17′31″N/8°46′38″W
Comporta	38°18′04″N/8°46′40″W
Aldeia do Meco	38°28′07″N/9°11′09″W
S. Pedro Moel	39°47′57″N/8°59′23″W
Duna de Quiaios	40°13′43″N/8°52′02″W
Duna de Mira	40°26′22″N/8°17′06″W

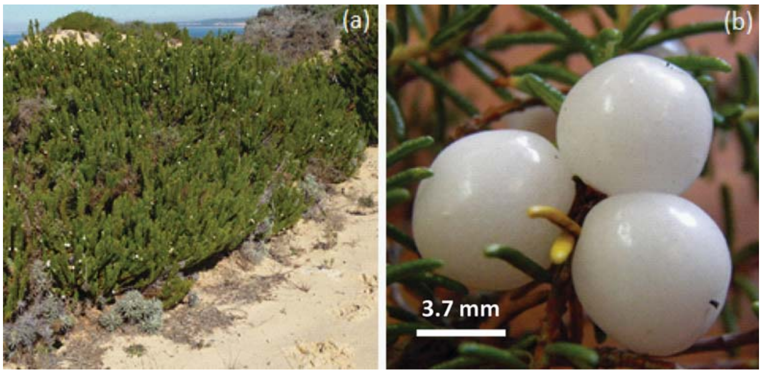

Fig. 1. (a) Female shrub with white or pink-white berry-like drupes and (b) White fruits.

After each collection date, seeds were separated from the pulp. Fruits were crushed gently to release the pyrenes (3 seeds), and washed free of fruit to remove any remaining mesocarp. The seeds were rinsed in tap water spread onto paper towels allowed to dry at room temperature and kept at 4°C for 19 months. The seeds collected in 2012 were then mixed with moisture sand and the mixtures stored at 4°C for 4 months (cold moist stratification).

2.2. Seed characterization

Area (minus any holes), major and minor diameter (reports the length of the longest and shortest line joining two outline points and passing through the centroid, respectively) and aspect (reports the ratio between the major axis and the minor axis of the ellipse equivalent to the object) of each seed collected in 2011 were measures through Image-Pro® Plus (version 7.0).

2.3. Germination experiments

Germination experiments were done with seeds from Duna de Quiaios in 2011, from Comporta in 2011 and Aldeia do Meco in 2011 and in 2012. Seeds were divided into four replications of 25 randomly selected seeds each and used for each pretreatment and location during all germination experiments.

2.3.1. Preparation and imbibition

Four replications of 25 seeds each were weighed and before initiating germination pretreatments seeds were surface-sterilized by soaking them in a 70% (v/v) ethanol for 5 minutes and commercial bleach (sodium hypochlorite) for 13 minutes [15]. After the disinfection, the seeds were rinsed in a stream of tap water and to see whether seeds could imbibe, each replicate was placed in tap water for 48 hours and weighed after imbibition.

2.3.2. Pretreatments

Experiments were modified from other methods [5, 15, 29].

Experiment 1: *Effects of acid scarification duration on germination responses*. The effect was investigated by exposing the seeds to concentrated sulfuric acid for 30, 60 and 120 minutes. Pretreatment were: H_2SO_4 (95–97%) in an ice bath for 30, 60 and 120 minutes, rinsed in a stream of tap water for 1 hour; then 5 minutes in $Ca(ClO)_2$ (3 g/L) completely dissolved in water with $Ca(OH)_2$ (3 g/L), and finally rinsed for 5 minutes in a stream of tap water.

Seeds with no acid scarification were used as a control.

Experiment 2: *Effects of acid scarification duration and gibberellic acid on germination responses*. Before pretreatment with GA_3, all seed replicates were exposed to concentrated sulfuric acid for 30, 60 and 120 minutes (Experiment 1). Seed replicates were then put into flasks containing 1000 ppm GA_3 for 24 hours in the dark.

Experiments were carried out in 90 mm diameter Petri dishes using 90 mm diameter Rundfilter Macherey-Nagel 816 filter papers that were kept moist with distilled water. Placement of Petri dishes in the germination chamber (Fitoclima D 1200 PL, Aralab) was completely randomized and seeds were placed with 8 hours dark at 15°C and 16 hours light (32 μmol m^{-2} s^{-1}) at 25°C for six months. In the last three months Petri dishes were kept in complete darkness due to the presence of green algae. Seeds were sprayed with propamocarbe fungicide (Previcur® N) and ciprodinil and fludioxonil fungicide (Switch®) every three weeks with one week interval between fungicides to control fungi. All Petri dishes of the experiment 1 and 2 were placed in a chamber in 6 and 22 of March 2013, respectively.

2.3.3. Data set and analysis

Rupture of the testa and germination were observed and registered every 2 weeks for 6 months, removing germinated seeds. Radicle emergence was the criterion used for scoring a seed as germinated. Seeds were considered to have germinated when a 1-mm-long or greater radicle emerged [23].

The effect of pretreatments on final rupture of the testa and germination percentages was statistically analysed using a two factorial (location and duration of pretreatment) design analysis of variance (ANOVA). All data were arcsine transformed prior to analysis in order to ensure homogeneity of variance. If ANOVA showed significant effects, means were separated with Tukey's multiple range test significant at alpha = 0.05. For the analysis of variance, statistical analysis software Statistix 9 (Analytical Software, Tallahassee, Florida) was used.

3. Results and discussion

3.1. Seed characterization

Different seeds characteristics differ significantly ($p < 0.000$) between sites (Table 2).

Similar results relating to the major and minor diameter were seen by other authors [10, 16, 21]. In relation to the seed aspect the results show that there are 4 groups defined by Tukey's test: Santo André and Dunas de Mira; Dunas de Mira and Duna de Quiaios; Duna de Quiaios, São Pedro de Moel and Comporta; São Pedro de Moel, Comporta, Aldeia do Meco and Pego, in which the means are significantly different between groups but are not significantly different from one another within group.

The lower values of area, major and minor diameter of the seeds probably indicates, according to other author [14], that seeds derived from old and large individuals with a higher competition by other species. These fragment populations invest much of their resources in growth and maintenance leaving little available for reproduction.

Table 2
Dry seed characteristics (area, major and minor diameter and aspect) from Santo André, Pego, Comporta, Aldeia do Meco, São Pedro de Moel, Duna de Quiaios and Dunas de Mira collected in 2011

Location	Area (mm^2)	Major diameter (mm)	Minor diameter (mm)	Aspect
S. André	12.1 A	4.7 A	3.2 A	1.5 A
Pego	11.4 A	4.3 B	3.3 A	1.3 D
Comporta	10.5 B	4.2 B	3.1 A	1.3 CD
Aldeia do Meco	9.0 CD	3.8 C	2.9 B	1.3 D
S. Pedro Moel	9.0 CD	3.8 C	2.8 B	1.3 CD
Duna de Quiaios	8.6 D	3.8 C	2.7 B	1.4 BC
Duna de Mira	9.5 C	4.1 B	2.8 B	1.4 AB
Mean	10.0	4.1	3.0	1.4
SEM	0.20	0.04	0.04	0.02
Prob. (F)	<0.001	<0.001	<0.001	<0.001

Means in the same column followed by different letters differ according to the Tukey´s test ($\alpha = 0.05$); SEM – Standard error of the mean; $N = 25$ seeds per location.

However, our experiment does not allow us to conclude if seed variability was only due to location or also owing to genotype, plant age or harvest date.

3.2. Germination experiments

3.2.1. Seed weight

The average weight of 25 dry seeds was 262.36 mg and the average weight of these seeds after imbition (48 hours) was 289.97 mg. The averages differ significantly among them ($p<0.000$, $N = 160$ petri dishes of 25 seeds each). Therefore, the thick woody endocarp enclosing seeds of *C. album* does not prevent imbibition of water. Because dormancy was not due to impermeability of the stony endocarp, seeds did not have physical or combinational dormancy.

For each experiment the average weight of 25 dried and thereafter imbibed seeds varied significantly ($p<0.05$) between sites. Similar results were seen by other authors [25].

3.2.2. Effect of pretreatments on germination

It was observed that rupture of the seed testa (coat) and rupture of the endosperm are two sequential events during the germination of *C. album*. These stages are followed by radicle emergence (Fig. 2).

Experiment 1: *Effects of acid scarification duration on germination responses*.

The testa rupture of *C. album* seeds differ significantly ($p<0.000$) between sites, regardless of duration of scarification, after 191 days of incubation. The highest average testa rupture (78.7%) was promoted on seeds from Aldeia do Meco in 2011 and the lowest average (18.2%) was observed on seeds from Aldeia do Meco in 2012. Regardless of collection site, acid scarification revealed significant effect ($p<0.000$) in percentage of ruptured seeds. The results also indicated the existence of a significant interaction ($p = 0.006$) between the collection site and acid scarification duration (Table 3). The average percentage of ruptured seeds from Duna de Quiaios increased as acid scarification duration was increased from 0 to 120 minutes. Also, the results show the highest percentage of ruptured seeds (79.0%) for the control on seeds from Aldeia do Meco in 2011 in relation to the same site but collection year in 2012 (6.0%). This suggests that collection year and storage condition can strongly affect seed testa rupture.

Significant differences ($p<0.000$) in percentage of germinated seeds were observed between sites, after 191 days of incubation. The results show that seeds collected from Aldeia do Meco in 2011 germinated better than all other seeds collected. Therefore, two groups of seeds could be distinguished: Aldeia do Meco in 2011 (15.7%), and all other seeds, Comporta in 2011 (2.2%), Aldeia do Meco in 2012 (1.2%) and Duna de Quiaios in 2011 (0.5%) with no significant differences between this 3 collection sites (second group). Also, the results showed no significant differences ($p = 0.588$) between 0, 30, 60 or 120 minutes. A significant interaction ($p = 0.042$) between the collection site and acid scarification duration was found in percentage of germinated seeds (Table 3). However, the results show that there are 3 groups defined by Tukey's test in which the means are not significantly different. The average percentage of germinated seeds from Aldeia do Meco 2011 with 30 minutes scarified (26.0%) was significantly greater than the value for control (7.0%) (Table 3). This suggests that application of sulfuric acid for 30 minutes is a promising method for improving seeds germination, as evidenced by another author [15]. Control seeds had

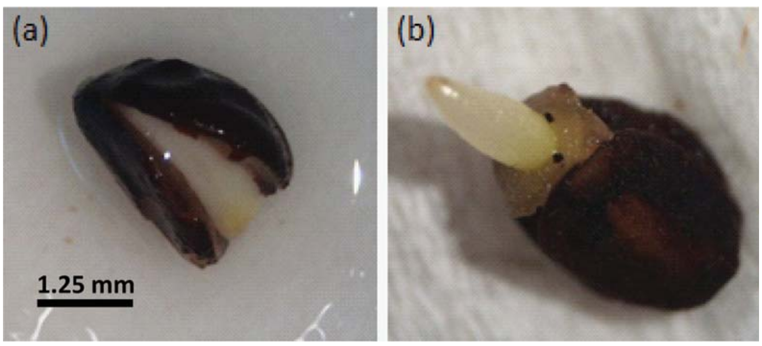

Fig. 2. Seeds during the germination. (a) Testa rupture and (b) Testa and endosperm rupture, as well as radicle emergence.

Table 3

Rupture of the testa and germination (radicle ≥ 1 mm) percentages per site (Comporta 2011, Aldeia do Meco 2011 and 2012 and Duna de Quiaios 2011) and acid scarification duration (30, 60, 120 minutes and control), after 191 days incubated

	Comporta 2011	A. Meco 2011	A. Meco 2012	D. Quiaios 2011
		Testa Rupture (%)		
Control	48.0 BCDE	79.0 AB	6.0 F	6.0 F
30 minutes	69.0 ABC	90.0 A	25.0 DEF	33.0 CDEF
60 minutes	61.0 ABCD	91.0 A	25.0 DEF	30.0 DEF
120 minutes	72.0 AB	55.0 ABCD	17.0 EF	47.0 BCDE
Mean	47.1			
SEM	7.22			
Prob. (F)	0.006			
		Germination (%)		
Control	4.0 BC	7.0 BC	0.0 C	0.0 C
30 minutes	1.0 C	26.0 A	1.0 C	0.0 C
60 minutes	1.0 C	18.0 AB	1.0 C	1.0 C
120 minutes	3.0 BC	12.0 ABC	3.0 BC	1.0 C
Mean	4.9			
SEM	3.01			
Prob. (F)	0.042			

Means in the same column or line followed by different letters differ according to the Tukey´s test ($\alpha = 0.05$); SEM – Standard error of the mean; $N = 4$ Petri dishes of 25 seeds each per location and acid scarification duration for the testa rupture and germination percentages.

germinated after 135 days and only 2.8% of non-treated seeds germinated after 191 days. All these results suggest that seeds were dormant and these are consistent with previous results from other authors in studies of *C. album* [9, 14, 15]. Most seeds reached their maximal germination at 163 days of incubation.

Experiment 2: *Effects of acid scarification duration and gibberellic acid on germination responses*.

Considering all seeds collected, 64.4% had testa ruptured, regardless of collection site and duration of scarification, after 175 days of incubation. The testa rupture differ significantly (p<0.000) between sites, regardless of duration of scarification. The highest average percentages of ruptured seeds were promoted on seeds from Aldeia do Meco in 2011 (Fig. 3). Regardless of duration of scarification, the highest average (92.7%) was promoted on seeds from Aldeia do Meco in 2011 and the lowest average (43.7%) was observed on seeds from Aldeia do Meco in 2012, while the seeds from Comporta and Duna de Quiaios had an average of 74.3% and 47.0%, respectively. Acid scarification followed by gibberellic acid revealed significant effect (p<0.001) in percentage of ruptured seeds, regardless of collection site. Significant differences ($p = 0.006$) in percentage of ruptured seeds were observed between the collection site and acid scarification duration. The curves varied in how quickly testa ruptured depending on the germination pretreatment and collection site and year (Fig. 3).

Significant differences ($p < 0.000$) in percentages of germinated seeds were also observed between sites, after 175 days of incubation. Germination was very low, with an average of 11.4% for the seeds used in experiment 2. However, this result shows that germination was significantly affected by the gibberellic acid, in relation to the experiment 1 with an average of 5.6%, after 191 days. This pretreatment, was highly effective in breaking dormancy and this pretreatment promote the best germination of *C. album* seeds collected from Aldeia do Meco in 2011 (Fig. 4). As for the first experiment two groups of seeds could be distinguished: Aldeia do Meco in 2011 (30.3%), and all others Comporta in 2011 (6.0%), Aldeia do Meco in 2012 (4.0%) and Duna de Quiaios in 2011 (5.3%) (second group). Also, the results showed no significant differences ($p = 0.107$) between 30, 60 or 120 minutes, followed by 1000 ppm of gibberellic acid. Therefore, this result suggests that application of sulfuric acid for 30 minutes is a promising method for improving seeds germination but now followed by 1000 ppm of gibberellic acid. Gibberellic acid was shown to enhance seed germination in several species of the family Ericaceae and to overcome physiological dormancy in

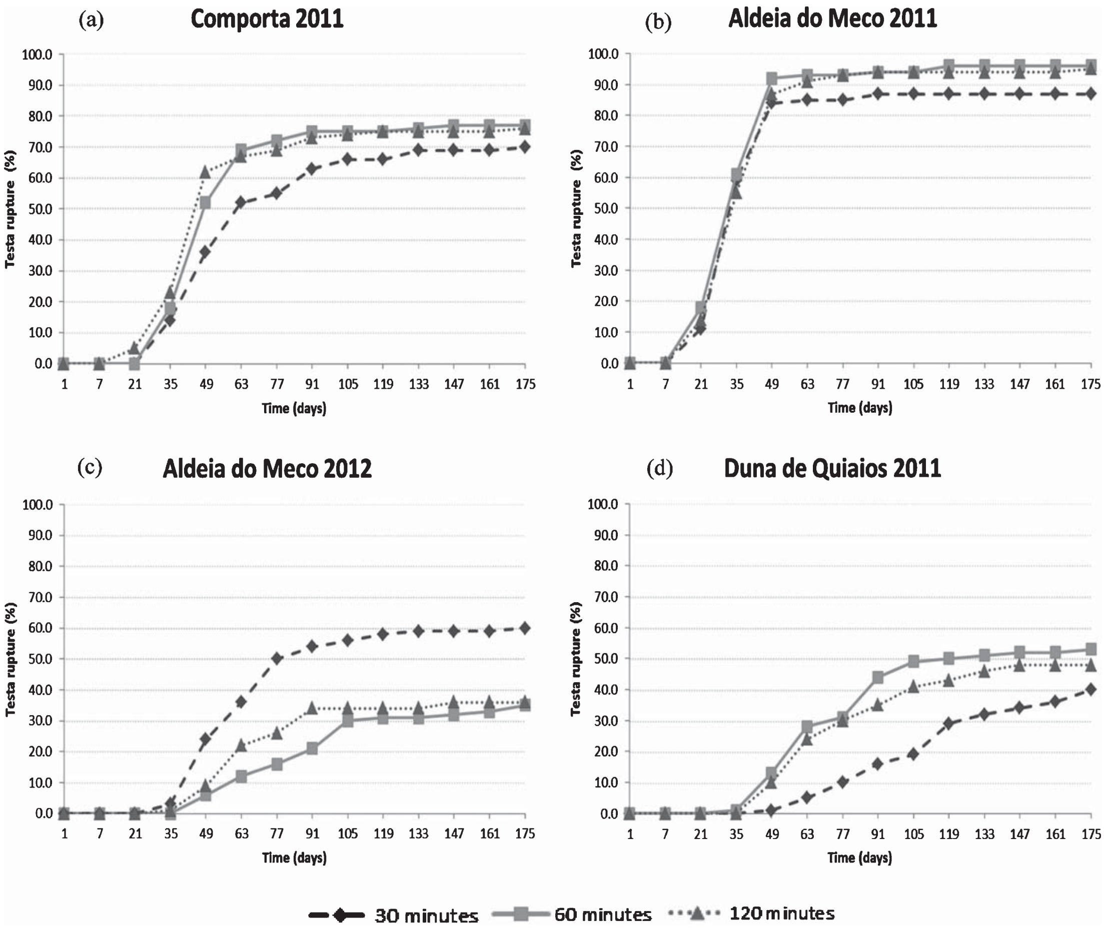

Fig. 3. The effect of acid scarification (30, 60 and 120 minutes) followed by 1000 ppm of gibberellic acid on the time courses of testa rupture of *C. album* seeds from Comporta 2011 (a), Aldeia do Meco 2011 (b), Aldeia do Meco 2012 (c) and Duna de Quiaios 2011 (d). The incidence of testa rupture was scored with time (days) after the start of incubation; $N = 4$ Petri dishes of 25 seeds each per acid scarification duration.

seeds with dormant embryos [5, 20, 27, 28]. Based on dormancy-breaking pretreatment and response to gibberellic acid to promote germination, *C. album* seeds had some type of physiological dormancy. A significant interaction ($p = 0.846$) between the collection site and acid scarification duration was found in percentage of germinated seeds. Many seeds collected from Aldeia do Meco in 2011 and Duna de Quiaios seem to continue their germination after 175 days of incubation, while most of the seeds reached their maximal germination before.

4. Conclusions

In the present study, there is site-to-site variation in the same year and year-to-year variation in a particular site in percentage of germinated seeds. Our results suggest that the overall probability of germination for a seed collected from Aldeia do Meco in 2011 is more than that of seeds collected from Comporta in 2011, from Duna de Quiaios in 2011 and from Aldeia do Meco in 2012.

New studies should be carried out for seeds from Aldeia do Meco, being necessary to evaluate the influence of the cold storage for a long period, so new tests should be carried out for seeds of a series of collects years from Aldeia do Meco exposed them to concentrated sulfuric acid for 30 minutes followed by 1000 ppm of gibberellic acid,

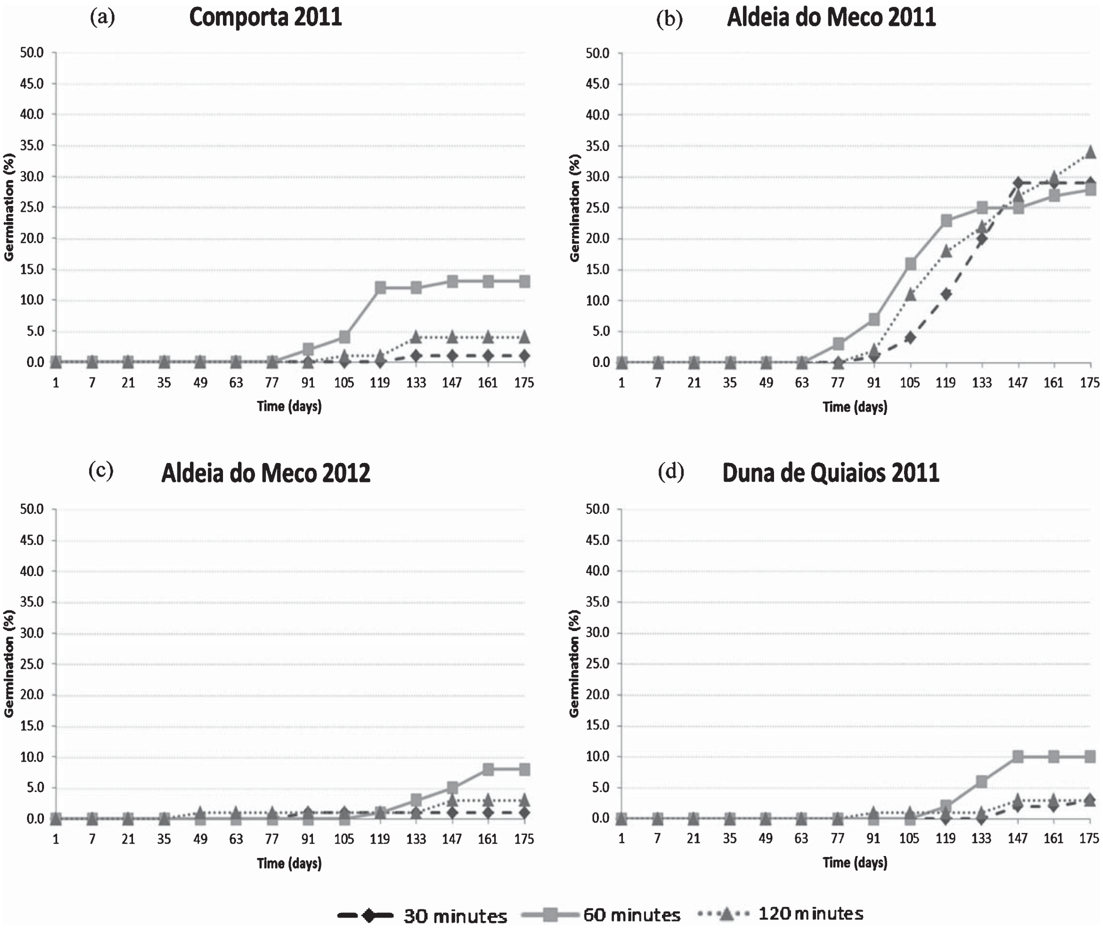

Fig. 4. The effect of acid scarification (30, 60 and 120 minutes) followed by 1000 ppm of gibberellic acid on the time courses of germination of *C. album* seeds from Comporta 2011 (a), Aldeia do Meco 2011 (b), Aldeia do Meco 2012 (c) and Duna de Quiaios 2011 (d). The incidence of germination was scored with time (days) after the start of incubation; $N = 4$ Petri dishes of 25 seeds each per acid scarification duration.

because collection year and storage condition can strongly affect seed germination. However, it should be noted that collection date and month can also affect seed germination, because the development of dormancy in some species is correlated with a decline in embryo water content, so in some species immature seeds may germinate faster than mature ones. The study of the influence of different concentrations and durations of imbibition seeds with gibberellic acid emerge itself, too, as pertinent. In addition, emerge as relevant the evaluation of seed viability, before the pretreatment.

As shown in this study rupture of the seed coat and rupture of the endosperm are two sequential events during the germination of *C. album*. These stages are followed by radicle emergence.

Our results also revealed that the seeds were found dormant, regardless of collection site or year and dormancy can be attributed to physiological inhibitory mechanisms of germination. Further, it is necessary to use histological techniques and measure seeds and embryos lengths before and during the germination studies to determine if fresh seeds of *C. album* have underdeveloped embryos and thus have morphological dormancy. According to the results seeds did not have physical or combinational dormancy.

Considering the 2800 seeds collected: 54.5% ruptured the seed coat and 7.7% germinated. These results clearly show that germination of *C. album* was very low. However, this study gives new important evidences that may play a key role in germination.

In conclusion, it is necessary to invest in new protocols to improve germination in order to obtain seedlings for the possibility of *Corema album* become a new berry crop.

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