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ORIGINAL RESEARCH ARTICLE

Effects of dormancy on seeds treatment germination of *Beta macrocarpa* achene

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ABSTRACT

Beta macrocarpa, Guss is an interesting species showing very low germination rates. The leading objectives of this work were to investigate the dormancy mechanism and to find methods to break dormancy in order to achieve rapid, uniform and high germination. Macro and micro-morphologic analyses were performed by stereo microscopy and scanning electron microscopy showed two fruit coats. The yellow external coat or persistent perianth coat (PPC) was accrescent with 5 erect segments contiguous to the operculum of the seed capsule. This coat forms spongy layers (50 to 300 µm thick) that could be eliminated manually. The narrow internal coat or pericarp or achene coat (AC) forms woody joined seed capsules, each presenting a pressed operculum that cannot be manually opened. This coat was not adherent to seeds and was composed of compressed cells (50 to 200 µm thick) which form pockets for salt cristal. Seeds were lentiform (1 to 2 mm diameter and 0.5 to 0.8 mm thick) and highly fragile. The embryo was whitish surrounded peripherally by the perisperm with two highly developed cotyledons and radical. Polyphenol concentrations in both coats showed that after 4 months of collection, total polyphenol concentrations were 4-fold higher in the pericarp than in the persistent perianth. However, after one year, this parameter decreases significantly in the pericarp, whereas, it increases to a larger extent in the perianth. Different germination tests indicated that the pericarp provides a chemical and a physical resistance to seed germination during the first 4 months of the experiment after collection. The chemical dormancy was released to higher levels of total polyphenol compounds that inhibited seed germination and seedling growth. However, the physical dormancy was associated with the hardness of this intern coat which caused a mechanical resistance to radicle emergence. After one year of storage, total polyphenol pericarp concentration decreased notably, and chemical resistance disappeared, whereas the physical one persisted. Consequently, one year of storage pericarp removal is sufficient to break this exogenous dormancy.

Keywords: *Beta macrocarpa*; Seeds; Germination; Treatment; Dormancy

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1. Introduction

Beta macrocarpa, Guss belongs to the family of Chenopodiacea and it has been used as a fodder crop as well as for human consumption. It is an annual herb that occurs in Portugal, Spain, Baleares, Canary Islands, Morocco, Algeria, France, Sicily, Greece, Cyprus, and Turkey[1]. This species was also recently introduced in Tunisia. Nevertheless, difficulties associated with the germination of this plant stand as obstacles to its success. This species is propagated by seeds which are enclosed into a woody/corky structure, the achene[2]. Currently, available germination protocols deliver low percentage, unsynchronized and lengthy germination. To date, the mechanism of achene dormancy for

this species is yet unknown.

However, successful reintroduction of a species using seeds needs knowledge of the germination requirements of seeds[3]. Understanding germination requirements is essential in the case of seeds that need a dormancy period.

According to Baskin and Baskin[4], dormancy can be caused by physical or physiological features or both. Koger *et al*.[5] declared that a dormant seed is one that does not have the capacity to germinate in a specified period under any normal physical environmental factors such as temperature, light, pH, and soil nature.

de Castro *et al*.[6] described that dormancy is caused by the requirement for a period of embryo growth and radicle emergence after the mature seed has been dispersed. In the same way, Derek Bewley and Black[7] consider a seed to be dormant if the only environmental factor preventing it from germinating is the accumulation of some toxic compounds.

Physical dormancy can be broken by scarification (nicking the seed coat or rubbing the seed with sandpaper) or soaking seeds in a weak-acid solution[4].

Physiological dormancy has been reported in Nyctaginaceae[8]. Germination of seeds has been examined in some species of Abronia[9]. Lee *et al*.[3] explained that the seeds of *Vaccinium oldhamii* exhibited physiological dormancy and chemical treatment can effectively break the dormancy of these plant seeds.

Yet, most publications on seed dormancy have not indicated the kind of dormancy that is being investigated. The objective of this work is to explain the *Beta macrocarpa* dormancy seeds mechanism and to propose solutions to break dormancy in order to obtain ideal seed germination.

2. Material and methods

2.1 Collection, storage and fruit characteristic

Beta macrocarpa dry fruits were collected in July 2020 from Soliman sebkha (a salt marsh 35 km southeast of Tunis) situated at 36°43.48 N, 10°21.00 E and they were stored at laboratory condition at 18–25 °C and 40%–50% relative humidity (RH). Twenty fruits were separated into perianth, achenes, pericarp and seeds for the determination of the weight of each part. Achenes were obtained by the manual removal of the perianth. Seeds were carefully removed once the pericarp was detached with pruning shears. Fruits, achenes and seeds lengths were measured, and their images were performed by means of a camera mounted on a stereo microscope LEICA S6E. Micromorphological analyses of *B. macrocarpa* fruit were selected on 10 carefully excised fruits. The microphotographs were taken using the scanning electron microscopy (FEI quanta 200, Eindhoven, the Netherlands).

2.2 Imbibition of seeds

To determine the permeability of coats fruit (perianth and pericarp) presented a barrier to water imbibition of seeds, the moisture content (MC) of seeds removed from imbibed (immersed in water for 10 days) and no imbibed fruit was determined. The MC was determined following the equation:

$$MC = \frac{\text{fresh weight} - \text{dry weight}}{\text{dry weight}} x100$$

The fresh and dry weights were based on the weights of three replicates of 10 seeds before and after oven drying.

2.3 Preparation and polyphenol content of plant extracts

2.3.1 Preparation of plant extracts for bioassay

For the determination of total phenol contents in perianth and pericarp, 1 mg of powder was homogenized with 10 mL methanol (2:8) under magnetic stirring at room temperature for 30 min. The extracts were kept for 24h at 4 °C and filtered through a Whatman N°4 filter paper. They were stored at 4 °C until analysis[10].

2.3.2 Total phenolic content

Phenolic content was assayed in both fruit coats using the Folin-Ciocalteu reagent, following Singleton's method slightly modified[11]. An aliquot (0.125 mL) of appropriately diluted sample extract was added to 0.5 mL of distilled water and 0.125 mL of the Folin-Ciocalteu reagent. After 3 min, 1.25 mL of Na_2CO_3 solution (7 g/100 mL) was added and the distilled water was then added to afford 3 mL of final

volume. The absorbance was measured at 760 nm, after incubation for 90 min at 23°C in the dark. Total phenolic content was expressed as mg gallic acid equivalents per gram of dry weight (mg GAE/g DW) through the calibration curve with gallic acid. The calibration curve range was 0–400 µg/mL. Triplicate measurements were taken on all samples.

2.4 Germination treatments

To determine the nature of the seed dormancy in *Beta macrocarpa*, germination of isolated seeds, seeds enclosed in intact achenes, seeds enclosed in de-coated achenes, seeds placed in contact of two fragments of perianth or pericarp (5 to 10 mg) leached or not in methanol solution were tested during the first four months or after 12 months of seed collection. Achenes were de-coated by detaching the pericarp with pruning shears at the radicle protrusion. All the seeds utilized for these tests were not undamaged. The methanol solution was employed with agitation for 3 days for the partial elimination of polyphenol components. All manipulations of seeds were placed on filter papers in 5 cm diameter plastic dishes in dark incubators maintained at the appropriate moisture and temperature regimes (13/24 °C). Four replicates (8 seeds or achenes each) were used.

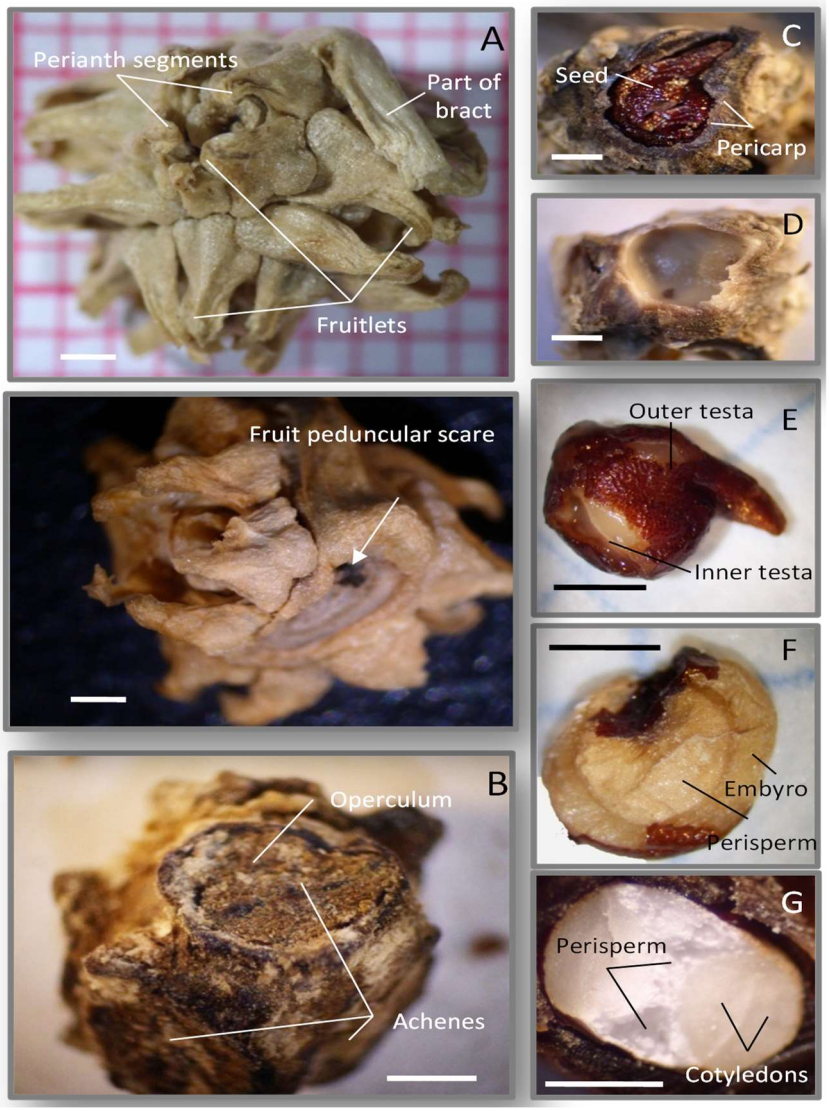

Figure 1. Structure of mature fruit and seed of *Beta macrocarpa*. **(A)** Dry fruit adhering in fruitlets, note that each fruitlet was covered with yellow swollen corky perianth and that perianth segments (5) were erect, longer and bent in fruitlet. **(B)** The base face of the fruit showing the fruit peduncular scare (B). Dry fruit after elimination of the persistent perianths, note narrow woody achenes closed with pressed operculum. **(C)** Seed capsule after operculum elimination and **(D)** Seed elimination. **(E)** The rounded face of the seed; note the rupture of the outer testa and the appearance of the inner testa. **(F)** The plate face of the seed after the elimination of the outer testa; note the central perisperm and the curved embryo which rounds the circumference. **(G)** General aspect of the seed sectioned transversally; note the farinaceous structure of the perisperm and the 2 cotyledons of the embryo. All bars = 1 mm.

After 12 days, the germination process of the seed was then considered to be completed when the young plant emerged and not completed when the germination was stopped after the emergence of the radicle or the embryonic shoot through the pericarp.

2.5 Statistical analysis

Means and standard deviations were calculated for total polyphenol and tannin concentrations. Separate one-way analyses of variance (ANOVA) were used to determine whether there were significant differences among cultivars and between raw and processed samples. A probability value of $P \leq 0.01$ was considered significant. The analysis was done using STATA 10-IC software (Stata Statistical Software: Release 10, StataCorp, College Station, TX, USA).

3. Results

3.1 Macro and micromorphology of *Beta macrocarpa* achene's

Beta macrocarpa mature dry fruit was a compound fruit known as cryptocarpi composed of 3 to 6 indehiscent fruitlets hidden by floral parts with a single bractlet at the base. Fruit diameter ranged from 6 to 9 mm and the weight ranged from 60 to 76 mg. Each fruitlet contains one seed and was enclosed within the persistent perianth coat (PPC) which was accrescent, swollen corky, hypogynous and scarious with 5 erect segments contiguous to the operculum of the seed capsule. Manual elimination of the PPC showed narrow woody joined seed capsules (achene), each presenting a pressed operculum that cannot be manually opened. Achene sections showed that the narrow achene coat (i.e., pericarp or internal fruit coat) became yellow in the zone adjacent to the seeds and was not adherent to the seeds. Electronic microscopy revealed that the PPC form spongy layers about 50 to 300 µm thick and the pericarp form compressed cells about 50 to 200 µm thick. These cells can form at some localities pockets for salt crystal (**Figure 1**).

Operculum removal with a pruning shear divulges lentiform seeds friable to the touch. They

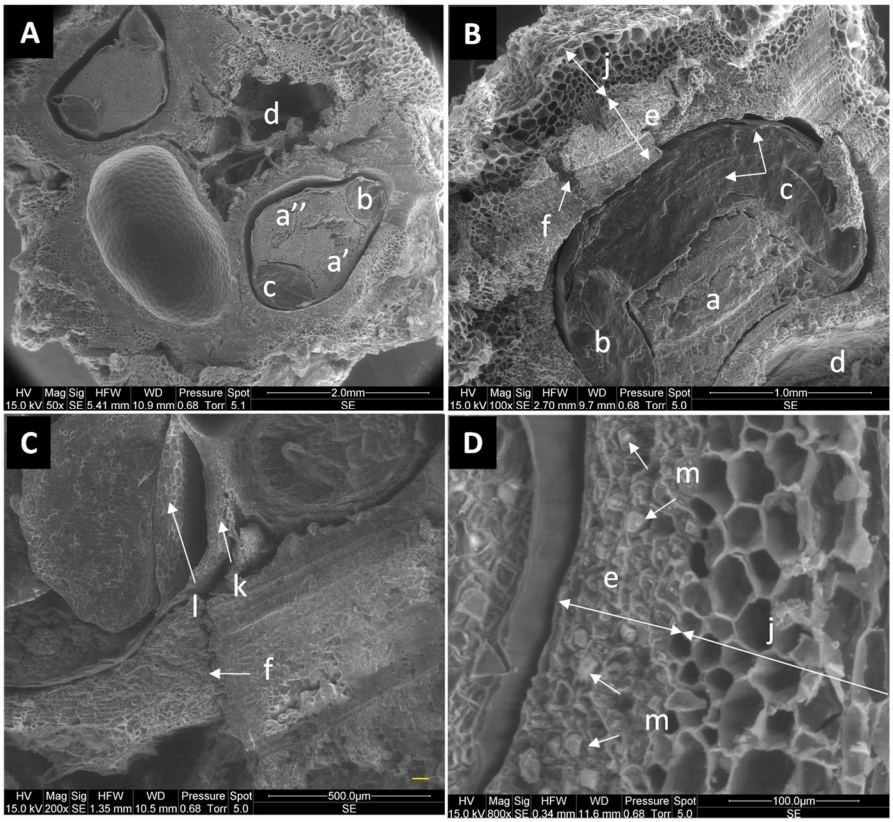

Figure 2. Photomicrographs of the fruit sections of *Beta macrocarpa* **(A)** transverse section of fruit parallel to the base face, note three seeds in which two were sectioned transversally with (a') plate face of the perisperm, (a'') rounded face of the perisperm, (b) radicle, (c) two cotyledons, and (d) fruit cavity connected to the fruit peduncular scare. **(B)** Longitudinal section of seed covered with fruit coats, note (a) perisperm, (b) radicle, (c) two cotyledons, (e) pericarp with (f) micro fissures and (j) persistent perianth. **(C)** The (k) outer testa and the (l) inner testa of the seed detached from a seed sectioned transversally and **(D)** The micromorphology of the persistent perianth coat and the pericarp, note the salt crystals.

showed two faces: a plate face adjacent to the operculum and a rounded face adjacent to the inner face of the seed capsule. The diameter of the seeds ranged from 1 to 2 mm and the thickness ranged from 0.5 to 0.8 mm. The seed coat was formed of two testas. The outer testa is dark brown and thick, whereas the inner one is light brown and thin.

The whitish embryo was surrounded peripherally by the perisperm and occupied the external area of the plate face of the seed. It exhibited varying degrees of curvature and was approximately 1.5 to 3 mm in length. It appeared with two highly developed cotyledons and a radicle. The radicle tends to occupy a peripheral position in the seed, which often produces a characteristic beaked (protrusion) asymmetry. The perisperm of *B. macrocarpa* seed was plentiful, whitish, farinaceous and more extended at the rounded face of the seed. It was densely packed with starch grains as indicated by a positive reaction with the PAS (blue-violet color with Lugol's solution) (**Figure 2**).

3.2 Polyphenol concentrations and identification in fruit coats

After 4 months of collection, the total polyphenols concentrations were 4-fold higher in the pericarp (2.17 ± 0.43 mg EAG/g DW) than in the perianth (7.43 ± 0.23 mg EAG/g DW). After one year, these concentrations decreased significantly (4.26 ± 0.28 mg EAG/g DW) in the pericarp, whereas it increased to a larger extent (7.16 ± 0.32 mg EAG/g DW) in the perianth (**Table 1**).

3.3 Germination

The percentage of germination observed after 12 days of imbibition remained unchanged even following 2 months of study. After 2, 4 or 6 months of collection, the germination rate of seeds separated from fruit reached 90%–95% (**Figure 3**). Nevertheless, they failed to germinate when they were preserved in fruits. The percentage of water uptake by non-scarified and scarified seeds was similar in both cases, about 80% of the water uptake occurred after

Table 1. Total polyphenol contents in fruit coats (perianth and pericarp) of *Beta macrocarpa* after 4 and 12 months of storage

	Perianth		**Pericarp**	
	4 months	12 months	4 months	12 months
Polyphenol contents (mg EAG/g DW)	2.17 ± 0.43	7.16 ± 0.32	7.43 ± 0.23	4.26 ± 0.28

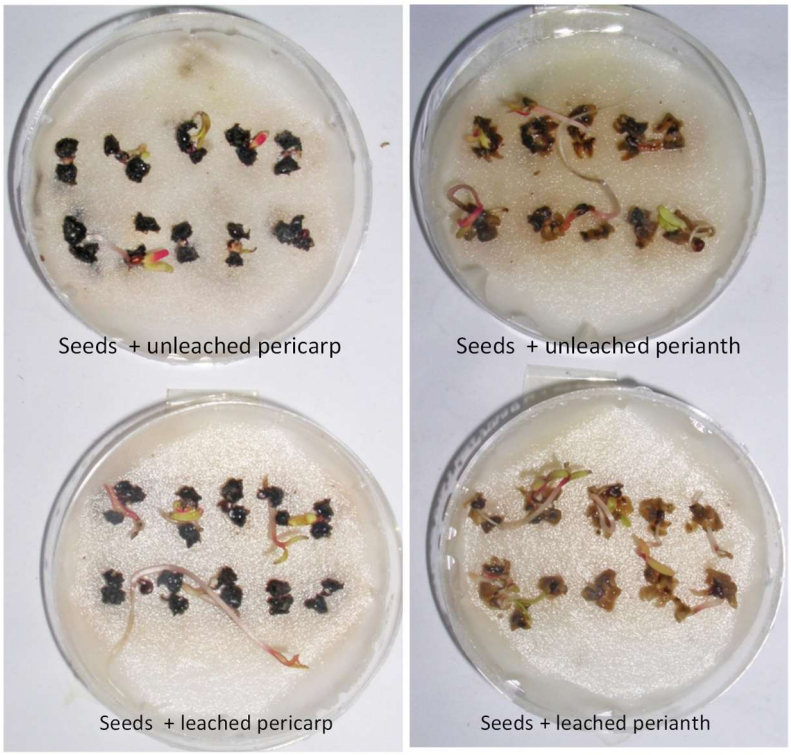

Figure 3. Twelve days after seed imbibition of *B. macrocarpa* placed in contact with leached and unleached pericarp or perianth. Seeds were stored for 4 months in laboratory condition and fruit coats were soaked for 72 h in methanol solution.

12 days of imbibition. Thus, the fruit coats of *Beta macrocarpa* did not act as a barrier to water diffusion into the seed. Physical removal of the pericarp (decoating) by cutting improved germination in terms of percentage of radicle emergence (10% to 15% showed radicle < 2 mm), however, the germination process stopped completely after this stage (no seeds had completed their germination). The most likely explanations for the inhibitory effects of fruit coats were that these coats contained inhibitors that exerted their effects on the embryo before or after germination initiation.

Fruit coats contained substantial concentration of polyphenol which can assess the role of phyto-inhibitors. Germination tests of seeds placed in contact with different parts of the fruit coats leached or not by methanol showed that seeds of *Beta macrocarpa* were markedly more sensitive when they were placed in contact with unleached pericarp (achene coat). In this case, nearly 50% of seeds started germination and no one had reached the seedling stage. Germination of seeds in contact with unleached pericarp was stopped after the emergence of radicle or embryonic shoot. With leached pericarp the percentage of initial germination increased, reaching 60% in which only 10% of seed had completed their germination. The percentage of initial germination of seeds placed in contact with an unleached perianth was not different from that of seeds placed in contact with an unleached pericarp (50%); however, the increase of the percentage of seeds that attempted the seedling stage to 20% indicated that the leaching treatment of perianth antagonized partially the inhibitor roles of polyphenol on germination. Leaching perianth resulted in a significant increase in the percentage of the initial germination (80%) and did not significantly change the percentage of the final germination. Thus, it is clear that the fruit coat of *B. macrocarpa* presented chemical dormancy (**Figure 4**).

Chemical dormancy disappeared fully after 12 months of storage with the germination of 90% of seeds placed in contact with unleached pericarp or unleached perianth. Nevertheless, the absence of germination of seeds preserved in intact achenes after the breaking of the chemical dormancy confirmed the presence of a physical dormancy which continued after one year of storage.

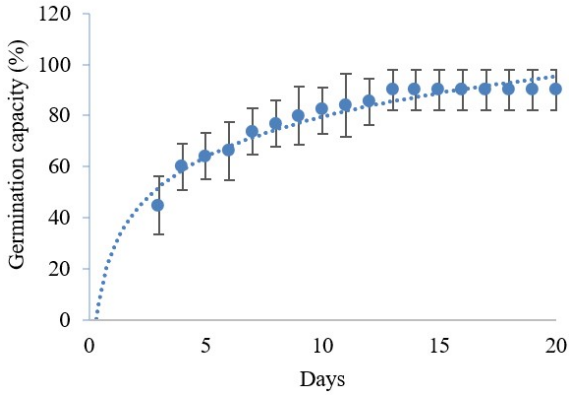

Figure 4. Seeds released germination from *B. macrocarpa* fruit envelopes.

4. Discussion

Isolated seeds of *B. macrocarpa* germinated immediately after collection. When they were enclosed in fruit, germination was totally inhibited throughout the 12-month duration of experiment. This result indicated a clear inhibiting effect of the fruit coat on seed germination. The partial removal of the pericarp around the seeds was effective at overcoming dormancy after 12 months of storage but not in fresh fruit (germination 15%). Based on this primary information, it has been concluded that fresh fruit coat provided a chemical and a physical resistance to germination. After one year of storage, the chemical resistance disappeared, whereas the physical resistance persisted. The fact that only the freshly separated coats (even when they were kept at a distance of 0.5 cm, not shown) still inhibited the germination of the embryos strengthened the existence of chemical resistance during the first 4 months of the experiment after collection. When seeds were placed in contact with an unleached pericarp or perianth they exhibited an initial emergence of only 50% of seedlings in which only 20% of seeds achieved their germination in contact with the perianth. Dorne[12] described that inhibitory effects on the germination of chenopodium bonus-henricus are related to the phenolic amounts in the seed coat. According to these authors, it appears that the total interruption of germination processes of the emerged seedling in contact to pericarp was probably related to its higher concentration in total polyphenol. Besides, the higher decrease (–53%) of the total polyphenol concentrations in the pericarp after one year of storage can explain the release of the chemical inhibition of this coat.

Subsequent leaching of polyphenol content from both coats highly improved the percentage of the initial germination (80% perianth and 60% pericarp), however, a slight percent of these seedlings achieved their germination (30% perianth and 10% pericarp). These results suggested that water, soluble phenolic components of pericarp and perianth inhibited the seed germination and the seedling growth of *B. macrocarpa*. The result found in this study is congruency with the data of Baleroni *et al*.[13] who found that some phenolic compounds significantly reduced hypocotyl and radicle length when compared with the control. They showed that ferulic and p-coumaric acids influence canola seed germination. Haddadchi and Gerivani[14] demonstrated that phenolic extracts of canola (*Brassica napus*) caused the reduction in germination, hypocotyl and radicle length of soybean (Glycine max).

Phenol components identified after 4 or 12 months of storage in the pericarp and in the perianth of the fruit of *B. macrocarpa* were the gallocatechin and the chlorogenic acid. The chlorogenic acid was linked with seed germination inhibition in *Artemisia herba alba*[15], its effect was strongly correlated to the decrease of the activities of glucose-6-dehydrogenase (G6PDH E.C 1.1.1.49) and 6-phosphogluconate dehydrogenase which are the key enzymes of pentose phosphate pathway[16]. Generally, the major phenolic compounds were reported as allelopathic. These compounds were inhibitory to spore germination, gametophyte development[16], seed germination and radical length of some species[17] especially the flavonoids (such as gallocatechin) were reported as phytoalexin against certain microorganisms. They have antibacterial and antifungal activity[17]. Thus, it seems that phenolic compounds in the seed coat of *B. macrocarpa*, especially in the pericarp, protect the fragile farinous seeds against the invasion of the microorganism and play an important role in regulating achene dormancy.

Research suggested that the phenol accumulation which was correlated with seed coat color played also a protective role in strengthening the plant cell walls during growth by polymerization into lignins[18] and caused a seed coat mechanical resistance to radicle emergence[19]. Our anatomical and chemical investigation of the fruit coat structure present evidence that the hard narrow pericarp, with its compact palisade layer woody structure and which was more concentrated in polyphenol than the yellow perianth is the main reason for the physically difficult germination of this species. It has been suggested that germination inhibition by the seed coat may be due to impermeability and (or) mechanical restraint[20,21]. However, there is considerable confusion in the literature concerning impermeability of seeds with "hard" seed coats, because the ability to take up (imbibe) water has not been tested in most of them. For example, *Opuntia tomentosa* seeds were reported recently to have a water-impermeable hard seed coat in combination with physiological dormancy[22]. For *B. macrocarpa*, despite the presence of the physical dormancy, the fruit coats in our species do not affect water absorption by the seed, as shown by the similar imbibition in scarified seeds than in non scarified seeds. It was evident from ultrastructure observation that the permeability of the pericarp was assessed by the existence of some micro-fissure that may act as a water channel. Thus, it seems that, in spite of the presence of an operculum in the *B. macrocarpa* achene, the fruit coats acted as a physical barrier that restricted the radicle growth. On the other hand, the seed coat helps protect the fragile embryo from mechanical injury and from drying out.

5. Conclusions

In conclusion, *B. macrocarpa* seeds had the chemical and mechanical exogenous dormancy released due to the essential presence of gallocatechin and chlorogenic acid in the pericarp. Further studies were needed to precise the mechanism of action of gallocatechin and chlorogenic acid in the regulation of the exogenous dormancy in this species.

Author contributions

Methodology: ABO; supervision: KM, MST and NL; conceptualization: NL; investigation: ABO and RBM; data curation: SR, OF and SD; project administration: NL; writing—original draft preparation: ABO and WAW; writing—review and editing: ABO and WAW. All authors have read and agreed to the

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Conflict of interest

The authors declare that they have no conflict of interest.

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