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Germination of Sardinian black and white *Vitis vinifera* seeds according to treatments and dormancy factors[1]

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ABSTRACT - Physiological dormancy of *Vitis vinifera* seeds jeopardises breeding programs and biodiversity evolution. To increase the knowledge on dormancy breaking, seeds of white and black Sardinian grape cultivars (*cvs*) were exposed to different pre-germination treatments. To shed light on the physiological and structural factors involved in seed dormancy, the contents of oil, abscisic acid, gibberellic acid, 3-indolacetic acid, condensed tannins, and total polyphenols were determined. In addition, sectioned seeds were observed by SEM to determine the morphological and anatomical characteristics. Dormancy break in white, but not in black grape seeds, occurred under almost all imposed pre-germination treatments. Among red *cvs*, only seeds from 'Cagnulari' germinated when kept at 25 °C. Chilling seeds of the white *cvs* 'Malvasia sarda' and 'Vernaccia di Oristano' for 30 d resulted in the most effective treatment. Compared to white *cvs*, seeds of red ones owned 7 times higher levels of abscisic acid however, gibberellic acid content resulted 4 times less. Concerning the coat characteristics, red *cv* seeds had a thicker cuticle (6–10 µm) than white (4–6 µm) ones, however the most significant diversities were found for the inner integument, where in addition to size variances, palisade cell wall were structurally different.

Key words: Hormones. Seed coat. Thermal treatments.

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INTRODUCTION

Vitis L. (the grape genus) consists of about 70 species in the *Vitaceae* family. The domesticated grapevine species *Vitis vinifera* L. is widely cultivated and represents the most economically important fruit crop in the world (GERRATH; POSLUSZNY; MELVILLE, 2015; WEN *et al.*, 2018). According to OIV data in 2020 (OIV, 2020.), the world's planted area is estimated at 7.3 million hectares. Within the EU, the latest available data for 2020 indicate an increase in the area under vines in France (797 kha, +0.4% /2019) and Italy (719 kha, +0.8% /2019). During the domestication process of *V. vinifera* subsp. *sylvestris* (C.C.Gmel.) Hegi, early viticulturists, selected traits related to fertility, productivity, berry size, sugar, and acid content (ZOHARY; HOPF; WEISS, 2012). This lasting selection process, based on phenotypic characteristics, such as hermaphroditism, has been the key for successful grape domestication and agricultural exploitation (BACILIERI *et al.*, 2013). Today, the global grape cultivar heritage consists of about 10,000 cultivars due to the long history of grapevine cultivation, somatic mutations, and vegetative propagation, as well as the creation of new hybrids and rootstocks (LAUCOU *et al.*, 2011). In the last few decades, *V. vinifera* breeding programs have implemented germination experiments aimed at singling out plants with resistance to biotic/abiotic stress. Reintroduction and/or reinforcement of the populations at risk of extinction have been the main goals for *V. vinifera* subsp. *sylvestris* (JI; WANG, 2013). Generally, *Vitis* seeds are physiologically dormant and require long periods of cold stratification before germination (ORRÙ *et al.*, 2012). Physical factors (temperatures, humidity, and light), together with chemicals, such as gibberellic acid (GA_3), may increase seed germination promptness (KIM *et al.*, 2015). However, viable seeds of some grape cultivars remain dormant even under suitable germination conditions and following appropriate dormancy breaking treatments. In addition to structural factors and embryo maturity, dormancy-specific genes and hormonal balance (ABA/GA) are also involved in primary dormancy (FINCH-SAVAGE; LEUBNER-METZGER, 2006; GRAEBER *et al.*, 2012). Concerning the influence of seed reserves on dormancy until recent reports, it has been a widespread opinion that their mobilisation occurred as a post-germination process (ZHAO *et al.*, 2018; GALLAND *et al.*, 2017). Concerning lipids, positive and negative correlations have been reported according to species (SATYANARAYANA; SUBHASHINIDEVI; ARUNDHATI, 2011). Normally, among seed reserves, lipids jeopardise water absorption and the unsaturated/saturated ratio and polarity of lipids, affects thermal time, especially at sub-optimal temperatures (BATOOL *et al.*, 2022). This effect has been attributed to the preservation of membrane functionality by polyunsaturated lipids (GONZÁLEZ BELO *et al.*, 2014). In addition, seed poly-embryony affects dormancy breaking, as evidenced by Bouquet (1980), who showed that poly-embryonic seeds were less prone to germinate compared to mono-embryonic ones and that chilling length influenced germination kinetics. The increasing need to attain crops resilient to climate change and the scarce information on seed germination physiology of Sardinia's grape cultivars has been the rationale for this work aimed at:

1 shedding light on seed germination physiology of seven native cultivars (*cvs*).

2 exploring the seed germination differences between white and red grape *cvs*.

3 stating oil content of native red and white grape seeds and corroborating its implication in seed germination physiology.

4 gaining knowledge on seed coat structural differences among native grape *cvs* and their effects on seed germination.

MATERIAL AND METHODS

- Chemicals, abbreviations, and factory codes:

Methanol (MeOH); Ethanol absolut (EtOH); Formic acid (FA); butylated hydroxytoluene (BTH) diethylether (DEE); AcCN Acetonitrile (AcCN); MilliQ wate (MQW); Vanillin (VA); Sodium Carbonate anhydrous (SC); Gallic Acid (GA); (Folin Ciocalteau reactive (FCR); Petrolium ether (PE); (+)-abscisic acid (ABA); 3-indolacetic acid (IAA); trans-zeatin riboside (CKY); giberellic acid (GA_3); hydrochloric acid (HCl); petrolium ether (PE).

Codes: MeOH (# 528101); EtOH (#3086052); FA (#405832); DEE (#P0441008); AcCN (#P0060228); SC (#479307); PE (#447836); FCR (#E463562); PE (#447821); HCl (#404161) from Carlo Erba, MI – Italy. GA (#G7384); VA (#GV1104); ABA (#90769); IAA (#45533); TZR (#Z0876); GA3 (#48880) from Sigma – Aldrich, S. Louis, MO – USA.

- Seed collection, cleaning, drying, and storage:

Seeds from four red (black) native *cvs* ('Carignano', 'Cagnulari', 'Bovale sardo', and 'Nieddera') and three white ones ('Moscato bianco', 'Malvasia sarda', and 'Vernaccia di Oristano') were attained from clusters harvested randomly near a grape catalogue vineyard of Fo. Re. S. T. A. S. (Agenzia Forestale Regionale per lo Sviluppo del Territorio e l'Ambiente della Sardegna) south-west Sardinia, Italy (39°08'98.33" N 8°49'48.69" E), managed under standard agronomic conditions with cordon-trained vines. Cluster

harvesting took place at the ‘N’ ripening index stage, according to Baillod and Baggiolini, (1993). Seeds were separated from berries by squashing the pulp on a sieve under a tepid water wash, screened, and only mature fully shaped ones were spread on an absorbing paper and kept to dry in the dark at room temperature (25 ± 2 °C and 40% relative humidity). Seeds samples are stored and available near the Centro Conservazione Biodiversità (CCB) (Supplementary Material Table 1).

- **Pre-germination treatments and seed germination trials**:

Following one week of drying at 20 °C, 18 uniform seed sets were prepared for each *cv* made up of three replicates of 20 seeds. Then, seeds of each replica were put with the ventral side down in 90-mm-diameter Petri dishes containing 1% agar water media. According to a 3-factor factorial experimental design including three pre-treatments (0, W, C), 7 *cvs*, and six thermal conditions. Dishes were first split into three groups (42 dishes each), where group ‘0’ was the control; group ‘W’ was subjected to a heat pre-treatment (25 °C); and group ‘C’ to a cold pre-treatment (5 °C). Each group contained 7 subgroups embodying the native *cvs*, and each subgroup was made up of six sets (sub-subgroups) of seeds, each linked to a different thermal regime. Throughout the experiment, the sub-subgroups of seeds of group ‘0’, and only following the three-month pre-treatment of those of group ‘W’ and ‘C’, were thermally kept at 10, 15, 20, 25, 30 °C and at a fluctuating temperature of 25/10 °C (12/12 h).

All sets were subjected to a cyclic 12 h dark/light period, and under thermal fluctuating conditions, the light period matched the high-temperature phase. Seeds were scored as germinated as soon as radicle emergence was visible (≥1 mm) and the trail was established as concluded when no additional seed germination was monitored within a two-week subsequent period. Then, to determine the remaining viability of the non-germinated seeds, a cut test was carried out discarding the soft, mouldy seeds that were considered non-viable.

- **Oil content quantification of seed**:

To determine the oil content, 20 g of one-week air-dried seeds of each *cv* were employed. Before extraction, seeds were treated in liquid nitrogen and then ice dried to remove water. Once dehydration was completed (water activity 0.2), seeds were ground by a ball-mill (Retsh Emax, Retsch GmbH, 42781 Haan, Germany) operating at 40 rpm for 20 min (particle size ≤0.4 mm), and oil extraction was performed according to the Soxhlet protocol with small adaptations. Briefly, 5 g samples of the dry ground matrix were placed in an extraction thimble and Soxhlet-extracted for 4 h using 200 mL of PE (40–60 °C). After extraction, most of the solvent was recovered by means of a rotavapor (R-300 Büchi, Cornaredo, Italy), and the remaining traces were removed by heating at 105 °C for 30 min (ventilated oven). Then, once cooled in a desiccator overnight, the extracted oil was weighed. For each *cv*, three extractions were performed, and the mean values ± SE are reported.

- **Extraction and cleanup of abscisic acid, 3-indolacetic acid, and gibberellic acids**:

- *Extract preparation*:

For each of the 7 *cvs*, 550 seeds (25–27 g) were employed to extract and quantify ABA, IAA, and GAs. In short, liquid nitrogen-frozen seeds were powdered as reported previously, and a matrix of 150 µm (max size) was gathered by colander sieving. Then, for each hormone, three replicates of 2 g were extracted twice at a 1:10 (w/v) ratio with cold (−20 °C) MeOH/FA/MQW (15:1:4 v:v:v) containing 1 mM·L^{-1} BHT as antioxidant. Each extraction was performed in the dark under shaking conditions (100 rpm) at 4 °C for 6 h. Supernatants, attained by centrifugation at 4 °C at 16,000 × *g* (Sovrall Super T 21, DuPont, Milan, Italy) for 15 min, were combined, and the remaining debris removed by vacuum filtration (GF10 and 0.22 µm, AMICON, INC, Beverly, MA, USA).

- *ABA, IAA clean-up*:

Concerning ABA and IAA a first extract-cleaning took place using a Sep-Pak Plus C18 cartridge (Waters, Milford, MA, USA, Cat #WTWAT-020515) according to the protocol of Dobrev and Kaminek (2020). Following this step, MeOH was withdrawn under vacuum at 40 °C with a rotavapor, while the aqueous FA residue was subjected to freeze-drying until dryness. Then, the dry powder was reconstituted in 6 mL of 1 M FA, and a further clean-up was performed with a water-wettable sorbent with reversed-phase and strong cation-exchange mixed properties (Oasis MCX 6ccVac, Waters, Milford, MA, USA, Cat #186000256). Following extract loading on the column, a first wash occurred with 6 mL 1M FA, and once the column was run dry, ABA and IAA were recovered in the eluate of a second wash with 6 mL of MeOH. Absorbance for IAA and ABA was attained by spectrophotometer (Agilent 8453, Agilent Tech. GmbH, Waldbronn, Germany) at 280 and 260 nm, respectively. Then, eluates were dried as reported previously and stored at −80 °C until hormone quantification was performed using competitive inhibition enzyme immunoassays.

- *GAs cleanup*:

Regarding Gas, the freeze-dried extract was dissolved in 2 mL aqueous MeOH (1:20 v:v) and managed with small changes, according to Urbanová *et al*., (2013). Briefly, the dissolved extract was loaded on an Oasis MCX cartridge, and 15 mL of MeOH/1 M FA/water (20:1:1 v:v:v pH 3) was used to wash the

column. Then, the run-through was loaded on a strong hydrophilic, reversed-phase, water-wettable polymer matrix (Supel-Select HLB- Supelco Analytical, Bellefonte, PA, USA, Cat #54182-U) and once the column run dry, Gas eluate was obtained by applying 3 mL MeOH/DEE (1:4 v:v). The collected fraction was dried as reported previously, and the dry residue was solubilised in 50 µL MeOH and brought to 3 mL with 25 mM NH4HCO3. Subsequent purification occurred by loading the solubilised residue on a column with a mixed hydrophobic and anion-exchange stationary phase (Oasis MAX Vac, Waters, Milford, MA, USA, Cat #186000371), which was previously activated with MeOH (3 mL), equilibrated with 25mM NH_4HCO_3 (6 mL), and after loading the extract, washed with AcCN (4 mL). Finally, GAs were eluted with 0.5 M FA in AcCN (6 mL) and dried, as reported previously. Dry extracts were stored at −80 °C until further use.

- *Pre-conditioning, activation, and equilibration of cleanup beads*:

Pre-conditioning of Sep-Pak Plus C18, Oasis MCX, and Supel-Select HLB beads (stationary phases) was achieved by activation and equilibration with 5 mL MeOH and 1 M FA, while the Oasis MAX bead was activated with MeOH (5 mL) and then equilibrated with 6 mL of 25 mM NH_4HCO_3. Resolution, selectivity, and dynamic capacity of each column was determined using ABA, IAA, TZR, and GA_3 standards. The most efficient elution profile for all clean-up steps was established. By varying the working parameters in screening experiments with the pure compounds, no glassware was employed, and runs were performed at 25 °C.

- *Abscisic acid, 3-indolacetic acid, trans-zeatin riboside and gibberellic acids quantification*:

The quantification of hormones was accomplished by specific competitive inhibition enzyme immunoassays (ELISA). The assay kits for ABA, IAA, and TZR were purchased from Agrisera, Vännär, Sweden (Cat #AS20-4392; #AS11 1749; #AS12 1844, respectively), while GAs were quantified as GA_3 using a kit from MyBioSource Com. San Diego, CA, USA (Cat # MBS9310617). The protocols were provided by the manufacturer. Samples/standards were prepared in duplicates and, when needed, extracts were diluted to obtain an O.D. read within the linear range of the standard curves, attained by plotting the log of the hormone concentrations vs the log of the O.D. Standard curves of each hormone were achieved using at least six concentrations and one blank. Absorbance was set at 450 nm for ABA and GA_3 quantification, while IAA and TZR reads were performed at 405 nm. To calculate the hormone concentrations, the average of the duplicate samples/standard O.D. readings were subtracted from the blank O.D. value. Substrate incubation time was established to attain an O.D. read >0.700 for the standard at its highest concentration.

- **Condensed tannins quantification**:

The seed matrix was attained as described previously, and the condensed tannins were quantified according to the vanillin-HCl (VA-HCl) assay, as reported by Broadhurst and Jones (1978), with a few revisions. For each analysis, 0.5 g of seed powder was extracted twice in a water bath at 35 °C with 10 mL of a 1% MeOH/HCl solution for 20 min. After each extraction, the solvent was collected by centrifugation (16,000 × *g* at 4 °C for 15 min). Then, the two supernatants were pooled and cleaned up by adsorption on a Sephadex LH-20 column (Sigma-Aldrich Cat #LH2050G). A column wash was performed with 50 mL of EtOH (95%), and the desorption of condensed tannins was achieved with 10 mL of aqueous acetone (1:1 v:v). The acetone was removed using a rotavapor at 30 °C, and the tannin-rich residue was liquid-nitrogen-frozen and ice-dried. To quantify the condensed tannins, the dry extracts were dissolved in 1 mL of aqueous MeOH (1:1 v:v), and 0.25 mL of it was pipetted into a 1 cm disposable plastic cuvette to which 1.5 mL of VA-HCl solution was added. The VA-HCl solution was prepared by mixing 4% (w:v) of VA in MeOH-HCl (12.5:1 v:v). Following mixing, the solution was left to react at room temperature for 30 min before absorbance measurement at 500 nm with a UV-visible spectrophotometer took place. Condensed tannin quantification is based on a standard calibration curve ($R2$ = 0.995) attained with 6 VA concentrations. The results expressed in mg equivalents of vanillin per gram of dry matrix (VAE/g DM).

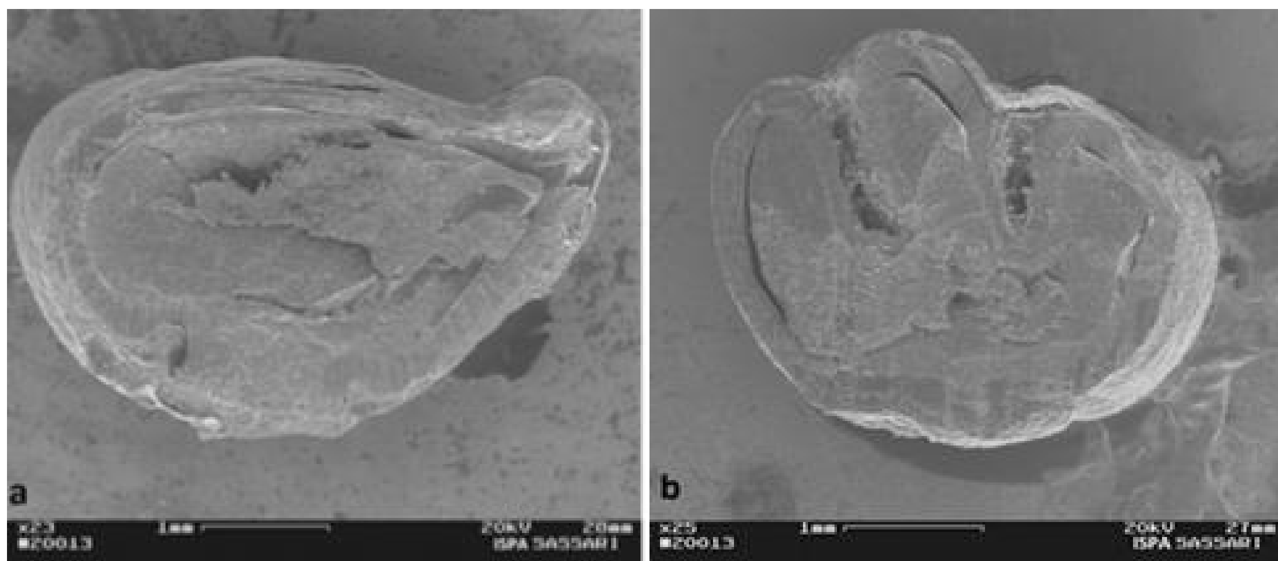
- ***Total polyphenol quantification***:

The polyphenol content was determined according to the Folin–Cocalteu protocol (SIN-GLETON; ORTHOFER; LAMUELA-RAVENTOS, 1999). Concentrations were calculated as equivalents of gallic acid, according to a six gallic acid concentration calibration curve ($R2$ = 0.998). The results expressed as mg equivalents of gallic acid per gram of dry matrix (GAE/g DM).

- ***Morphological and anatomical observations***:

Scanning electron microscopy (SEM) was employed to study grape seed morphological and histological characteristics. Seeds were prepared as described earlier, up to the removal of the pulp-debris. At that point, no drying occurred, but seeds were whipped with absorbing paper, and cold stored (8 °C and 60% RH) until use, which occurred within seven days following removal from the berries. For each *cv*, 36 seeds (three replicates of 12) were

Figure 1 - Micrograph of a longitudinal (a) and median transversal (b) section of a grape seed magnified at ×23 and ×25, respectively



randomly gathered from a 100 homogenous sub-sample of seeds. Then, by using a manual tissue chopper (TC-2; Sorvall, Newtown, CT, USA), six seeds of each replicate were sectioned longitudinally and the remaining cross-sectioned in the median area, as shown in Figure 1a, b. Sectioned seeds were treated with liquid nitrogen, freeze-dried, and mounted on aluminum stubs (25 mm) using glue and silver colloidal conductive paste. Afterwards, they were gold-coated with a sputter coater (S150 A, Edwards, MI – Italy), securing a uniform 200 nm gold layer. Observations took place under high vacuum conditions with an SEM (DSM 962, Zeiss, Austria) at 20 KV at different magnifications (×20 - ×5000). For each specimen, structural observations were performed, and the thickness of coat tissues was measured in four signposts, indicated as S1, 2, 3, and 4 (Figure 3a). For the medium integument, additional data were recorded concerning the number of pits/100 µm2 (Figure 5a, b, c). Then, all recorded data were statistically analysed.

- **Data analysis**:

Normalised values of all data were analysed using the Shapiro - Wilk test. Arcsine-transformed germination percentages were analysed using ANOVA and a subsequent Fisher's Least Significant Differences (LSD) post hoc test. Data were graphed using Sigma plot 11.0 (Systat Software Inc., London, UK), while all statistical analyses were carried out using the software Statistica 7.0 for Windows (Software Statsoft Release 7). Measurements of scanning electron microscopy observations were performed with the software Quartz PCI Imaging version 10 (Quartz Imaging Corporation, Vancouver BC, Canada).

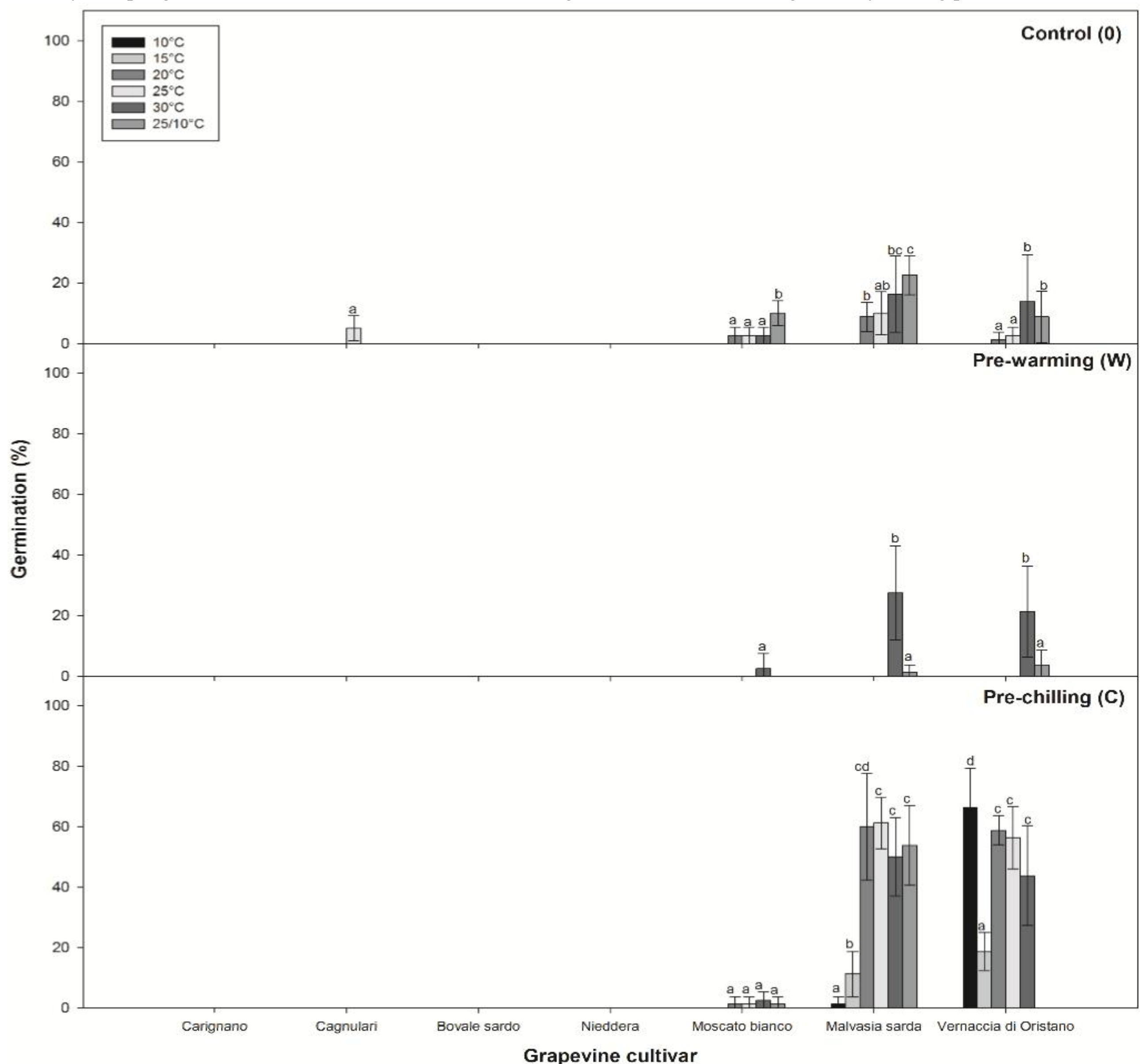
RESULTS AND DISCUSSION

The experiments carried out applying different thermal pre-germination treatments (0, W, C) to establish the role of various ecological conditions in breaking seed dormancy evidenced a different behaviour between white and red grape seeds. Indeed, seeds from white *cvs* germinated under almost all imposed pre-germination treatments and thermal conditions, reaching the highest degree of germinated seeds. As for the seeds kept under control (0) thermal conditions (Figure 2a), germination rates were generally low and monitored only at temperatures ≥20 °C. Concerning the white *cvs*, low values were recorded for 'Moscato bianco' kept at 20, 25, and 30 °C (2.5%), while higher percentages were monitored at 25/10 °C (10.0%). As for 'Malvasia sarda,' germination was positively correlated with the temperature rise, with percentages of about 8.8, 10.0, and 16.0% at 20, 25, and 30 °C, respectively. For this *cv*, the highest value (23%) was recorded following intermittent warming (25/10 °C). Finally, 'Vernaccia di Oristano' was characterised by the highest germination percentages at 30 °C (14.0%), followed by 8.8% at 25/10 °C, while the lowest values were recorded at 20 (1.3%) and 25 °C (2.5%). Concerning the red *cvs*, only seeds from 'Cagnulari' germinated, although at a very low rate (5.0%) at 25 °C (Figure 2a). As for the warming (W) pre-treatment effect on subsequent dormancy breaking during the different thermal regimes, at 30 °C, 'Malvasia sarda' and 'Vernaccia' di 'Oristano' had a higher germination percentage compared to the same thermal regime applied in the control (Figures 2a, b). However, the same pre-germination treatment negatively

affected the dormancy break in all subsequent thermal regimes, and it is likely that a second dormancy phase was induced by intermittent warming (Figure 2b). The chilling (C) pre-germination treatment was the most effective in terms of dormancy release during all subsequent thermal regimes, and seeds of the three white *cvs* showed a significantly higher germination ability ($P < 0.05$) (Figure 2c). As for 'Malvasia sarda', germination percentages of 1.3 ± 2.5 (10 °C), 11.0 ± 7.5 (15 °C), 60.0 ± 18.0 (20 °C), 61.0 ± 8.5 (25 °C), 50.0 ± 13.0 (30 °C), and 54.0 ± 13.0 (25/10 °C) were recorded. Likewise, high germination percentages were also observed for 'Vernaccia di Oristano' seeds, i.e., 66.0 ± 13.0 (10 °C), 19.0 ± 6.3 (15 °C), 59.0 ± 4.8 (20 °C), 56.0 ± 10.0 (25 °C), and 44.0 ± 17.0 (30 °C), while this was the only white *cv* that did not germinate under the alternating temperature regime. Among the white *cvs*, 'Moscato bianco' had the lowest germination rate, with percentages of 1.3 ± 2.5 (20, 25, 25/10 °C) and 2.5 ± 2.9 at 30 °C, and no germination occurred at 10 and 15 °C.

The oil content in fresh seeds of red native cultivars ranged between 10.16 and 11.42%, and in white cultivars, it ranged between 8.14 and 9.79%. In the dried seeds, the

Figure 2 - Effect of pre-germination treatments on the seed germination rate of seven native grape cultivars: (a) germination rate at constant thermal regimes (10, 15, 20, 25, 30 °C) and intermittent warming (25/10 °C) control (O); (b) germination rate as influenced by a 30-day heat pre-germination treatment at 30 °C (W); (c) rate of germinated seeds following a 30-day chilling pre-treatment at 5 °C



percentages ranged from 16.66 to 18.72% and from 13.13 to 15.80% in the red and white *cvs*, respectively (Table 1). The water content ranged between 34 and 36%, and differences among *cvs* were not significant. Concerning the oil content, red *cvs* had significantly more oil than white *cvs*, and 'Bovale sardo' had the highest content (11.42; 18.72%), while 'Carignano' had the lowest content (10.16; 16.66 %). 'Moscato bianco' was the white *cv* with the highest oil content (9.79; 15.80%), and 'Malvasia sarda' had the lowest content (8.14; 13.13%). According to the statistical analysis, less significant differences occurred when extraction was performed with fresh seeds, albeit, in both fresh and dry seeds, the highest and lowest oil contents matched the same *cvs* (Table 1).

With respect to ABA, the values ranged between 0.85 and 0.08 ng·mg^{-1} DW, thus indicating a broad variability among the studied *cvs*, with the highest value recorded in 'Carignano' and the lowest in 'Malvasia sarda' (Table 2). In general, the red *cvs* had a significantly higher content than the white ones. For IAA, the figure differed with a less clear difference between red and white *cvs*, but again the lowest concentration occurred in seeds of a white *cv* ('Moscato bianco' 0.04 ng·mg^{-1} DW) and the highest in red ones ('Bovale sardo' 0.23 ng·mg^{-1} DW). Trans-zeatin riboside (TZR), a cytokine representative, was detected at very low concentrations in all *cvs* (0.003 to 0.010 ng·mg^{-1} DW), and the highest concentrations were found in 'Malvasia sarda' and 'Vernaccia di Oristano'. Among the red *cvs*, only 'Cagnulari' had a concentration similar to that of the white ones (Table 2). The content of gibberellic acids, expressed as GA_3, was highly variable (0.04 to 0.18 ng·mg^{-1} DW), with white *cvs* having the highest concentrations while among the red ones, 'Cagnulari' had the highest concentration.

Following the extraction of total polyphenols from seeds of different white and red grape *cvs*, significant differences were observed, with the highest

Table 1 - Water and oil content (%) in seeds of seven Sardinian grape cultivars belonging to *Vitis vinifera* ssp. *vinifera*[X]

Grape cultivars Red *cvs*	H_2O (%)	Oil in fresh seeds (g·100g^{-1})	Oil in dry seeds (g·100g^{-1})[y]
Bovale sardo	35	11.42 a	18.72 a
Cagnulari	34	10.35 ab	17.25 b
Carignano	36	10.16 b	16.66 c
Nieddera	36	10.42 ab	17.36 b
White *cvs*			
Malvasia sarda	35	8.14 c	13.13 e
Moscato bianco	35	9.79 b	15.80 c
Vernaccia di Oristano	35	9.09 bc	14.66 d

[X] Values are means of n = 3 and different letters within each column indicate significance at P < 0.05 according to Tukey's studentised range test. [Y] Dry seeds refer to seeds that were one-week air dried + freeze-dried up to a value of 0.2 water activity (aw)

Table 2 - Concentrations of abscisic acid, 3-indolacetic acid, trans-zeatin riboside and gibberellic acids in seed-extracts of seven Sardinian grape cultivars[X]

Grape cultivars	ABA[Y] ng·mg^{-1} DW	IAA ng·mg^{-1} DW	TZR ng·mg^{-1} DW	GA_3 ng·mg^{-1} DW
Carignano	0.85 ± 0.08 AZ	0.15 ± 0.04 AB	0.004 ± 0.002 B	0.05 ± 0.01 C
Cagnulari	0.25 ± 0.05 C	0.10 ± 0.03 B	0.008 ± 0.003 AB	0.08 ± 0.02 B
Bovale sardo	0.55 ± 0.05 B	0.23 ± 0.03 A	0.003 ± 0.002 B	0.05 ± 0.03 BC
Nieddera	0.62 ± 0.09 B	0.20 ± 0.04 A	0.003 ± 0.002 B	0.04 ± 0.01 C
Moscato bianco	0.12 ± 0.04 D	0.04 ± 0.02 C	0.008 ± 0.003 AB	0.09 ± 0.02 B
Malvasia sarda	0.08 ± 0.02 D	0.09 ± 0.02 B	0.010 ± 0.002 A	0.18 ± 0.03 A
Vernaccia di Oristano	0.12 ± 0.05 D	0.09 ± 0.02 B	0.009 ± 0.002 A	0.11 ± 0.02 B

[X] Hormone concentrations determined according to specific competitive inhibition enzyme immunoassays (ELISA) following extraction and clean up; n = 5. [Y] Abscisic acid (ABA); 3-indolacetic acid (IAA); trans-zeatin riboside (TZR); gibberellic acids (GA3); dry weight (DW). [Z] Values are means of n = 3 and different letters within each column indicate significance between values at $P < 0.01$ according to Tukey's studentised range test

concentrations in black *cvs* 'Nieddera' and 'Bovale sardo' (0.62 and 0.55 GAE·g^{-1} DW, respectively) and the lowest detected in white *cv* 'Malvasia sarda' (Table 3). Lower concentrations than 'Bovale sardo' and 'Nieddera' were recorded by all other *cvs*.

By observing the median-transversal section of the seeds (Figure 3 b), five distinct tissues were observed corresponding to: 1) cuticle and epidermis (Cu, Ep); 2) outer integument (Oi); 3) medium integument (Mi); 4) inner integument (Ii) and centrally, 5) the endosperm and nucellus (En, Nu) (Figure 3b). In Figure 3a on the ventral and dorsal side (Vs and Ds), the raphe (R), karina (Ka), and fossette (Fo) of the seed are denoted and four marks, (S1, 2, 3, and S4) indicate the sites where tissue thicknesses were measured. In Figure 3b and in Fig. 4a, clear anatomic differences among coat tissues are evidenced.

The cuticle (Cu) resulted amorphous, covering the whole seed, and significant size differences were recorded among grape *cvs* (Table 4). In all *cvs*, the epidermis was composed of a monolayer of rectangular cells, and the thickness of the cell layer differed among *cvs* (Table 4).

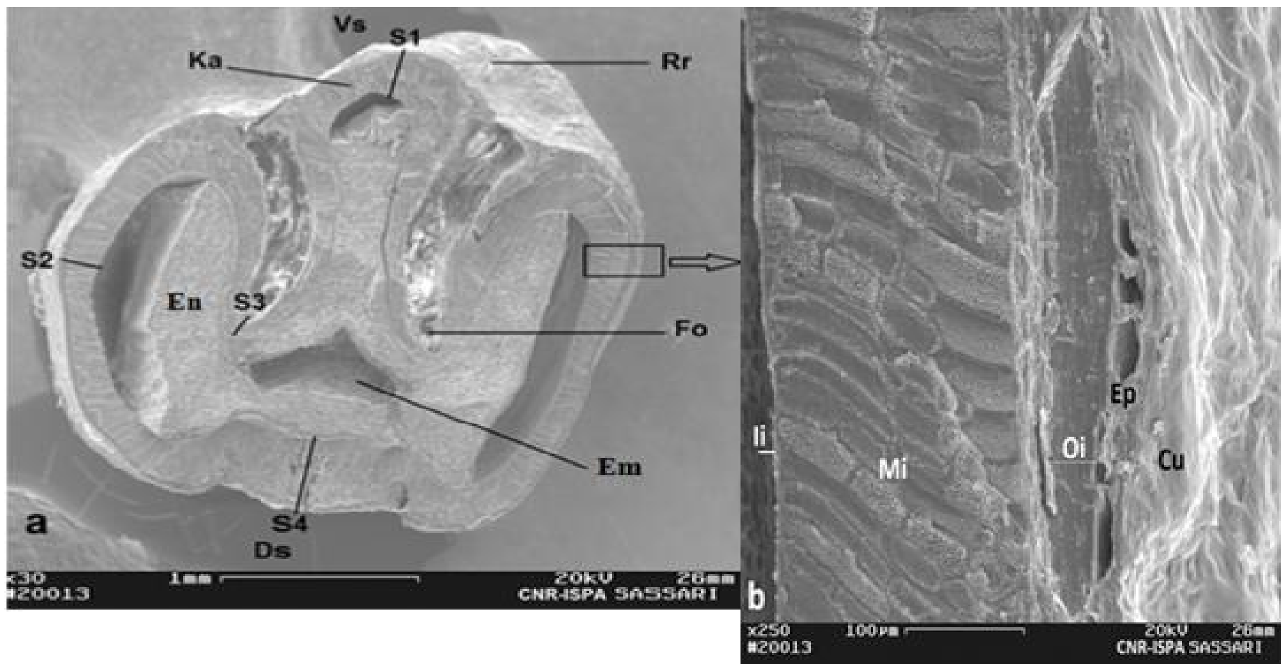
In some *cvs*, most of the epidermis cells collapsed. Concerning the outer integument (Oi)

Table 3 - Content of total polyphenols and condensed tannins in extracts of seeds from seven Sardinian grape cultivars[X]

Grape cultivars	Total polyphenols (GAE·g^{-1}DW)[Y]	Condensed Tannins (VAE·g^{-1}DW)[Y]
Carignano	0.15 ± 0.05 BC Z	0.005 ± 0.001 D
Cagnulari	0.25 ± 0.05 B	0.008 ± 0.001 B
Bovale sardo	0.55 ± 0.05 A	0.003 ± 0.002 D
Nieddera	0.62 ± 0.09 A	0.003 ± 0.002 D
Moscato bianco	0.12 ± 0.04 C	0.008 ± 0.001 B
Malvasia sarda	0.08 ± 0.02 C	0.010 ± 0.001 A
Vernaccia di Oristano	0.12 ± 0.05 C	0.009 ± 0.002 AB

[X] Red *cvs*: 'Carignano', 'Cagnulari', 'Bovale sardo', and 'Nieddera'. White *cvs*: 'Moscato bianco', 'Malvasia sarda', 'Vernaccia di Oristano'. [Y] GAE: Gallica Acid Equivalents; VAE: Vanillin Acid Equivalents; DW: Dry weight. [Z] Values are means of n = 3 and different letters within each column indicate significance between values at $P < 0.01$ according to Tukey's studentized range test

Figure 3 - (a) SEM (x30). transversal grape seed section indicating the dorsal side (Ds), ventral side (Vs), fossetta (Fo), raphe (R), karina (Ka), endosperm (ES), and embryo (Em), thickness records S1, S2, S3, and S4 (a). Marked area in Fig. 3a (x250), indicating the cuticle (Cu), epidermis (Ep), outer integument (Oi), medium integument (Mi), inner integument (Ii) (b).



(Figures 3b; 4a), 3–4 layers of cells were observed in all *cvs* with no size or thickness differences among them, while on the dorsal side (Ds; S4), cell size was greater than on the ventral side (Vs; S1) (Table 5). The median integument (Mi) was set up by two layers of palisade cells with thick walls and tightly packed (Figures 3b; 4a).

The walls bordering the inner integument (Ii) were always thicker than those of the Oi. Lengthwise, on the internal side of the palisade cells, three different patterns of structures were observed (Figure 5a, b, c).

According to the number of pits, the *cvs* were ranked in three groups (Table 6). The Ii structure showed three layers, namely two externals of tinny rectangular-shaped cells, one bordering the Mi and the other the endosperm (Es), while in the middle a differentiated untidy structure was observed (Figure 4b). The Ii structure and size were similar for all *cvs*. The embryo was located in the central area of the seed, and due to dehydration, the endosperm was shrunken and detached from the Ii (Figure 3a).

The data reported in Tables 4 and 5 account for the sizes recorded in the signposts (S) marked in Figure 3a, magnified in Fig. 3b and Fig. 4a. Significant differences were observed among *cvs*, especially for the Cu, Ep, and Mi records. Indeed, the Cu thickness in ‘Carignano’, ‘Bovale sardo’, and ‘Nieddera’ in S1 and S4 were between 6 and 13 µm, whereas in S2 and S3, the values were much lower (between 1 and 7 µm). The Cu size of ‘Cagnulari’ and ‘Moscato bianco’ seeds in S2 and S3 was similar to those of the 3 previous reported *cvs*; however, the S1 and S4 values were significantly lower (between 3 and 8 µm). The smallest Cu widths in S1 and S4 were recorded in the ‘Malvasia sarda’ and ‘Vernaccia di Oristano’ seeds (between 1 and 2 µm) (Table 4). Concerning the Ep size, values recorded in the four signposts of the same cv were equal, and *cvs* were arranged into two groups according to epidermis thickness: one group with values between 1 and 5 µm (‘Carignano’, ‘Bovale sardo’, and ‘Nieddera’) and a second one with values slightly greater (2 to 6 µm) observed in ‘Malvasia sarda’, ‘Vernaccia di Oristano’, ‘Cagnulari’, and ‘Moscato bianco’ (Table 4). Concerning the outer integument (Oi), the S4 records were the greatest for all *cvs* and ranged from 45 µm in ‘Malvasia sarda’ to 51 µm in ‘Nieddera’ (Table 5). The values in S1 ranged from 25 to 32 µm in ‘Nieddera’ to 32 µm in ‘Carignano’; the values recorded in S2 and 3 were similar, with a maximum of 26 µm in S2 and a minimum of 16 µm in S3.

Figure 4 - (a) SEM (×500), median transversal section of a grape seed coat evidencing the measurements of the cuticle (Cu), the epidermis (Ep) the outer integument (Oi), me-dium integument (Mi), and inner integument (Ii) (a); SEM (×5000), inner integument evidencing the three layer tissue structure (b)

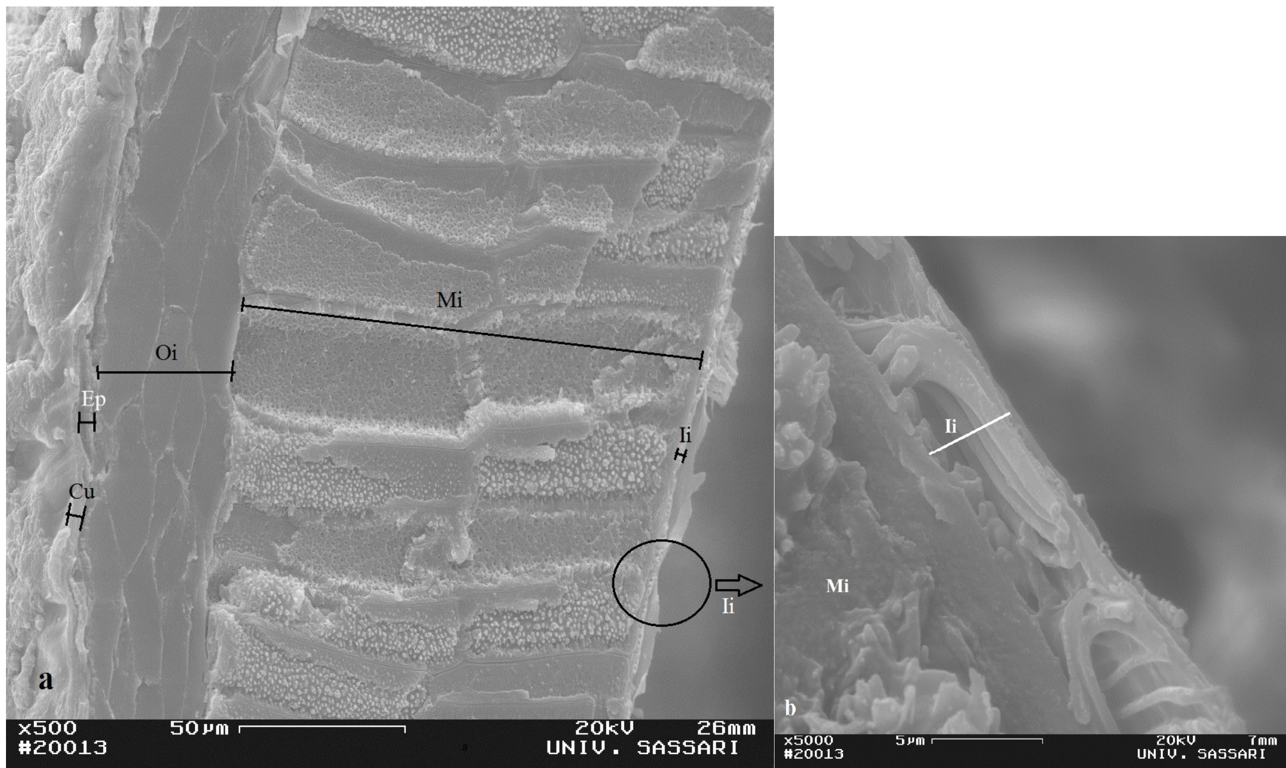
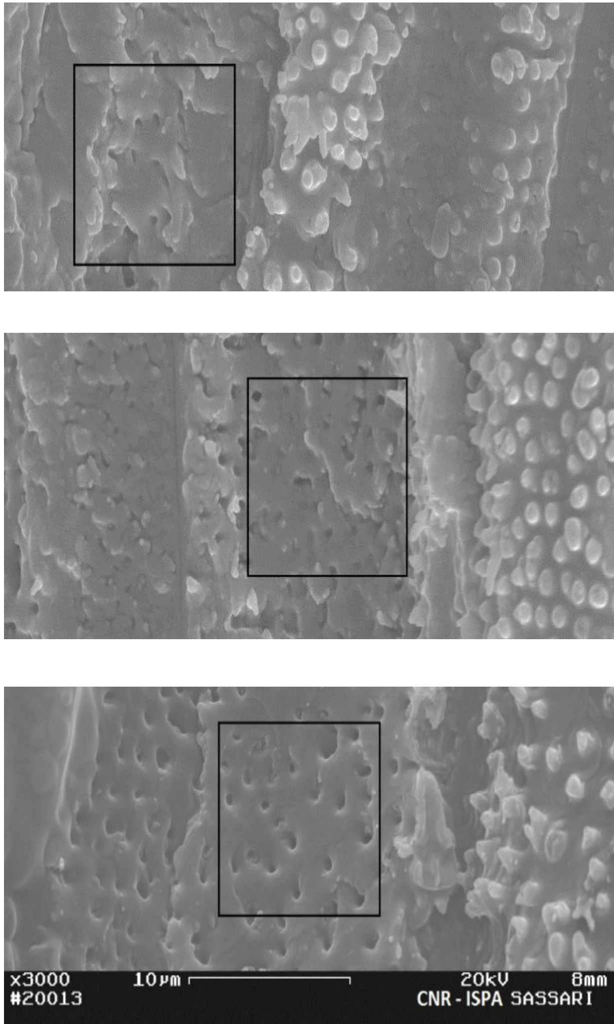


Figure 5 - SEM (×3000), medium integument (Mi) palisade evidencing cell-wall differences (a, b, c); The marked squares (10 µm2) were used to determine pit density



The medium integument (Mi) resulted in the most consistent structure in all *cvs* and accounted for more than 80% of the seed coat. Differences in thicknesses among signposts and *cvs* were significant, with S4 being the most consistent, with a maximum of 210 µm in 'Cagnulari' and a minimum of 175 µm in 'Bovale sardo'. The lowest values recorded in S1, S2, and S3 were those of 'Malvasia sarda' and 'Vernaccia di Oristano'. Values in S1 and S2 were similar for 'Moscato bianco', 'Nieddera', and 'Cagnulari' (between 150 and 125 µm) with a thinner size in signpost S3 (from 99 to 102 µm) (Table 5).

The inner integument (Ii) represents the coat structure in contact with the endosperm, acting as the transfer tissue for assimilates/water moving from the palisade cells to the nocellus and endosperm. This tissue was thin in all *cvs* with values similar to Ep, and no significant differences were observed among *cvs* or signposts (Tables 4 and 5).

According to the pit number and wall roughness recorder in 10 µm^2 squares (Figure 5a, b, c), the grape *cvs* were ranked into three groups (Table 6). This parameter was not dependent on grape colour, and 'Carignano', 'Bovale sardo', and 'Moscato bianco' palisade resulted in a quite smooth surface and a pit density ranging from 18 to 25 in a 10 µm2 square surface (Figure 5c). In the second group, only one red grape *cv* ('Cagnulari') was included, and its pit density ranged between 12 and 15, with a surface evidencing some wrinkled areas (Figure 5b). The third group, composed of 'Nieddera', 'Malvasia sarda', and 'Vernaccia di Oristano', had a Mi with a low pit density (2 – 6) and an extended wrinkled surface (Figure 5a).

Considering all results on the dormancy breaking of seven Sardinian grape *cvs* subjected to different

Table 4 - Cuticle and epidermis size in four signposts on the median transversal section of grape seeds from seven Sardinian cultivars [X]

Coat Tissue	Cuticle[y] (µm)				Epidermis[y] (µm)			
Signpost	S1	S2	S3	S4	S1	S2	S3	S4
Carignano[z]	9 ± 3 AB	4 ± 3	3 ± 1	10 ± 2 A	3 ± 2	3 ± 1	3 ± 1	3 ± 2
Cagnulari	5 ± 2 B	4 ± 2	2 ± 1	6 ± 1 B	4 ± 1	3 ± 1	3 ± 2	4 ± 1
Bovale sardo	9 ± 2 A	4 ± 1	3 ± 2	10 ± 3 A	3 ± 2	3 ± 1	3 ± 1	3 ± 2
Nieddera	9 ± 2 A	4 ± 2	3 ± 2	10 ± 2 A	3 ± 2	3± 1	3 ± 1	3 ± 2
Moscato bianco	5 ± 2 B	4 ± 2	2 ± 1	6 ± 2 B	4 ± 1	3 ± 2	4 ± 2	4 ± 2
Malvasia sarda	3 ± 1 BC	3 ± 2	2 ± 2	4 ± 1 C	4 ± 1	4 ± 2	4 ± 2	4 ± 2
Vernaccia di Oristano	3 ± 2 BC	3 ± 3	3 ± 2	4 ± 2 C	4 ± 2	4 ± 1	4 ± 2	4 ± 1

[X] Signposts S1-4 evidenced in Figure 3a [Y] Cuticle (Cu) and epidermis (Ep) evidenced in Figure 3b; signposts S1-4 shown in figure 3a [Z] Data were obtained by measuring the size using the software Quartz PCI Imaging version 10 and values are means of n = 18 records ± SE (µm). Different letters within each column indicate significance between values at $P < 0.01$ according to Tukey's studentised range test

Table 5 - Size of the outer, medium and inner integuments in the four signposts on the median transversal section of grape seeds from seven Sardinian cultivars[XY]

Coat Tissue	Outer integumenty(µm)				Medium integument (µm)				Inner integument (µm)			
signpost	S1	S2	S3	S4	S1	S2	S3	S4	S1	S2	S3	S4
Carignano	32 ± 5 a	22 ± 2	21 ± 2	48 ± 6 ab	130 ± 25 b	120 ± 20 b	110 ± 15 a	180 ± 22 bc	4 ± 2	4 ± 1	4 ± 2	4 ± 2
Cagnulari	28 ± 2 a	23 ± 3	22 ± 2	52 ± 3 a	152 ± 18 a	122 ± 28 b	99 ± 15 b	210 ± 10 a	4 ± 2	5 ± 1	4 ± 2	4 ± 2
Bov. sardo[Z]	30 ± 3 a	21 ± 4	20 ± 5	49 ± 3 a	133 ± 15 b	120 ± 10 b	100 ± 16 b	175 ± 28 c	4 ± 1	4 ± 2	4 ± 2	4 ± 2
Nieddera	25 ± 1 b	22 ± 4	19 ± 3	51 ± 5 a	148 ± 25 a	122 ± 30 b	102 ± 23 b	200 ± 10 a	4 ± 2	3 ± 2	4 ± 2	4 ± 2
Mosc. bian.	28 ± 2 a	22 ± 4	19 ± 2	49 ± 3 a	150 ± 21 a	130 ± 19 a	100 ± 21 b	205 ± 16 a	4 ± 2	4 ± 1	4 ± 1	4 ± 2
Malv. sarda	26 ± 3 a	21 ± 2	20 ± 2	45 ± 6 b	110 ± 16 c	100 ± 10 c	95 ± 8 bc	190 ± 10 b	4 ± 2	4 ± 2	4 ± 2	4 ± 2
Verm.Ori.	28 ± 5 a	20 ± 2	19 ± 3	48 ± 6 ab	121 ± 20 c	100 ± 22 c	98 ± 11b	185 ± 21 bc	4 ± 1	4 ± 2	4 ± 2	4 ± 3

[X] Outer integument (OI), Medium Integument evidenced in Figure 4a; Inner integument in Figure 4b; Signposts S1-4 shown in Figure 3a. [Y] Data were obtained by measuring the size using the software Quartz PCI Imaging version 10 and values are means of n = 18 records ± SE (µm). Different letters within each column indicate significance between values at $P < 0.05$ according to Tukey's studentized range test. [Z] Bovale sardo (Bov. sardo); Moscato bianco (Mosc. bian); Malvasia sarda (Malv. sarda); Vermentino di Oristano (Verm. Ori.)

Table 6 - Grouping of Sardinian white and red grape cultivars according to SEM observations on the palisade cell structural characteristics of the seed median integument[X]

Pit range (n°/10 µm^2)	Groups						
	16-28			11-15	1-8		
cultivars	Malvasia sarda W[Y]	Vernaccia di Oristano W	Moscato bianco W	Cagnulari R	Nieddera R	Carignano R	Bovale sardo R
Pit density[z]	25 ± 3 A	21 ± 2 A	19 ± 3 A	13 ± 2 B	5 ± 3 C	3 ± 2 C	3 ± 2 C
Surface characteristic	Smooth	Smooth	Smooth	Fairly wrinkled	Very wrinkled	Very wrinkled	Very wrinkled

[X] Scanning Electron Microscopy observations (x3000) n= 4 at the S4 signpost of 36 median traversal sections (Fig. 5a, b, c). [Y] Cultivar colour: white *cvs* (W) or Red (R) ones. [Z] Mean value of pits ± SE in 144 observations of palisade cells in the medial integument

thermal treatments and the data on seed characteristics, a clear picture of the studied white *cvs* was outlined, while for red ones, this research provides new data on their chemical, hormonal, and anatomical characteristics. Concerning the oil extracted from the grape seeds, the significant difference found between white and red *cvs* in our study cannot be attributed to environmental factors (HERTEL; EDWARDS, 2011) since grape clusters were harvested in the same experimental orchard, seeds employed for the analysis were carefully selected, and floaters discarded. This difference should rather be attributed to interspecies genetic determinants, as has been reported for grapes and other crops (PÉREZ-NAVARRO *et al*., 2019), or to differences in the embryo–endosperm seed ratio (MIRAY *et al*., 2021). The percentage of oil detected in the autochthonous grape *cvs* was below that reported for oil-crop seeds (38–53%) used to study the effect of oil reserves on seed germination (GONZÁLES-BELO *et al*., 2014). According to this study, the quantity of oil did not affect the germination time, in that the hydro-time to germinate in seeds with high oil content may be reached by absorbing low quantities of water. However, the reduction in the germination thermal time has been positively correlated to the ratio of the saturated/unsaturated fatty acids (FA) in the oil (GONZÁLES-BELO *et al*., 2014). According to several studies on the chemical composition of grape seed oil, up to 85–89% of the fatty acids are mono- to poly-unsaturated and as a general role white *cvs* have a higher percentage than red *cvs* (PÉREZ-NAVARRO *et al*., 2019). Thus, since unsaturated FAs preserve seed membrane characteristics and water potential, it is likely that dormancy breaking in seeds from white *cvs* kept at 30 °C following heat pre-treatment is related to high levels of unsaturated FA. This statement is supported by the fact that in the control, thermal regimes from 20 °C promoted dormancy breaking in all white *cvs*, while among the same thermal regimes applied after the heat pre-treatment, seed of white *cvs* germinated only by the thermal regime at 30 °C. Notably, at 30 °C, 'Moscato bianco' had values similar to the control, while 'Malvasia sarda' and 'Vernaccia di Oristano' germinated at a higher percentage compared to the control. However, intermittent warming decreased the germination percentage, suggesting the induction of secondary dormancy in all white *cvs* (BUIJS, 2020). Considering the effects of the

pre-treatments on dormancy breaking of seed from red *cvs*, it is clear that the treatments had no effect, or as for 'Cagnulari', had negative consequences. According to the analyses performed on the seeds before starting the germination experiments, a remarkable difference between the coat characteristics of the white and red *cvs* was evidenced by SEM observations. In particular, cuticle and medium integument sizes resulted larger in red *cvs*, and differences were also evidenced for the palisade cell structure. It is well documented that the coat structure affects germination promptness (KELLY; VAN STADEN; BELL, 1992); thus, it is likely that, compared to the white *cvs*, the coat structure in red *cvs* affected the hydro-time by jeopardising, together with the oil content, the water permeability (SMȲKAL *et al*., 2014). To support this statement, Walker *et al*. (1999) evidenced that in *Vitis* spp., during seed development, the palisade is directly involved in the assimilate translocation from the chalaza to the developing storage tissues via the inner integument transfer cells, regulating the delivery rate of assimilates to the nucellus and endosperm. In addition, seed imbibition rate depends upon the characteristics and number of plasmodesmata that connect the palisade cell symplasts and the intensity of cracks in the palisade layer, as reported by Chen *et al*. (2019), for seeds of 'Caragana' spp. Since in our observations we did not found cracks in the palisade tissue but a positive correlation between the number of plasmodesmata in the palisade and the percentage of germinated seeds occurred, we feel confident in attributing to these structural difference one of the factors that contributes to the results attained by the germination test. Additionally, the high levels of the hormones ABA and IAA and the low levels of GAs detected in red grape seeds are key factors involved in the dormancy break (CHOHAN; DHILLON, 1976). Concerning total polyphenols and condensed tannins, both are mainly located in the seed coat, and controversial effects on seed germination have been reported for different crops (CAVALLARO *et al*., 2021; DE ALMEÌDA *et al*., 2014). Most research reports a significant decrease in these chemical classes during seed stratification, but only recently by leaching carob seeds evidence was provided on their direct effect on breaking dormancy (CAVALLARO *et al*., 2021). Based on our results, red *cvs* were richer in polyphenols (TPC) compared to white ones, except for 'Carignano', while condensed tannins were lower in red ones. Even if we speculate that the total polyphenols may be among the factors that affected seed dormancy breaking, it is un-likely that they played a predominant role as reported for carob since, among the red *cvs*, only 'Cagnulari' germinated at 25 °C under control conditions, and its TPC was higher than 'Carignano' and all white *cvs*. Concerning the condensed tannins, white *cvs* had the highest levels; thus, it is likely that they are not among the seed germination determinants.

CONCLUSIONS

The results of the present research establish that the dormancy of seeds from red grapes is not interrupted by any of the pre-treatments (warm/cold) and that among the white grapes used, two ('Vernaccia di Oristano' and 'Malvasia sarda') have similar germination behaviour, while 'Moscato bianco' acts differently and seems less influenced by the pre-treatments, especially by heat. Secondary dormancy was induced by applying the intermittent warming thermal regime following heat pre-treatment. The main factors inhibiting germination of red *cvs* are likely to be related to coat-imposed dormancy, especially to the cuticle and the palisade structure and size, as well as the high levels of ABA, IAA, and low levels of GAs. The best results were attained by the cold pre-treatment applied to the seeds of white *cvs*, and both polyphenols and condensed tannins seem not to affect seed dormancy directly.

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