

Communication

Hydrogen Peroxide Imbibition Following Cold Stratification Promotes Seed Germination Rate and Uniformity in Peach cv. GF305

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Abstract: (1) Background: Peach cv. GF305 is commonly used in breeding programs due to its susceptibility to numerous viruses. In this study, we aimed to achieve a methodology for rapid and uniform seed germination of peach cv. GF305 in order to obtain vigorous seedlings; (2) Methods: A combination of cold stratification and H₂O₂ imbibition was tested on peach seeds with or without endocarp. In addition, the levels of non-enzymatic antioxidants ascorbate and glutathione as well as the hormone profile in seedling roots and shoots were determined; (3) Results: We found that H₂O₂ imbibition of peach seeds without endocarp after 8 weeks of stratification increased germination rate and resulted in seedlings displaying good vegetative growth. The H₂O₂ imbibition also affected the levels of ascorbate, glutathione, and the phytohormones abscisic acid and jasmonic acid in peach seedlings; (4) Conclusions: Although stratification periods of 12 weeks have been previously established as being appropriate for this cultivar, we have been able to reduce this stratification time by up to 4 weeks, which may have practical implication in peach nurseries.

Keywords: ascorbate; endocarp removal; hydrogen peroxide; glutathione; peach; phytohormones; seed germination; stratification



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1. Introduction

In stone fruit species, stratification (moist chilling of seeds) has been described as the most widely method to break seed dormancy and promote germination. Stratification simulates winter conditions keeping seeds chilled for 3 to 4 months [1]. In order to reduce this waiting period, the application of chemicals and the mechanical removal of the seed coat have been widely used in breeding programs [2]. The germination process is associated with many molecular, metabolic, and cellular events enabling radicle emergence and seedling establishment [3,4]. In both dormant and non-dormant seeds, the crucial role of phytohormones regulating seed dormancy breaking and germination has been long established, with reactive oxygen species (ROS) and hence the antioxidative metabolism closely linked [4]. ROS control many different processes in plants via redox-sensitive proteins that act as sensors and messengers of different regulatory pathways [5]. Seed germination must be included among these processes, with the antioxidative metabolism playing a key role [4,6]. However, the biochemical basis of seed dormancy regulation is still poorly understood [7].

Hydrogen peroxide (H₂O₂) has been described as an enhancer of seed germination in many species [3,4]. Different mechanisms have been suggested to explain the H₂O₂ stimulation of seed germination, with the following being the most common: the production of O₂ for mitochondrial metabolism and respiration as a consequence of H₂O₂ scavenging [8], the facilitation of seed cracking, the oxidation of germination inhibitors [9],

and the activation of redox-sensitive proteins, inducing changes at proteome, transcriptome, and hormonal levels [4,10]. In this sense, the decrease of abscisic acid (ABA) levels or its transport impairment from cotyledons to the embryo, as well as the mobilization of seed storage proteins, have been suggested as possible mechanisms underlying seed germination promotion through H_2O_2 [10]. In stone fruit seeds, ABA is the main hormone involved in seed dormancy, and a significant decrease in ABA has been recorded as the stratification time increases [1,11]. Moreover, in pea seeds, a role of H_2O_2 in orchestrating the interplay among phytohormones and the cellular redox state leading to seed germination and seedling establishment has been reported [3,10].

Stimulated germination by exogenous H_2O_2 has been reported on endocarp-less seeds of several *Prunus* species when applied before stratification. In this sense, a significant increase in the percentage and speed of seed germination by H_2O_2 was described in the wild almond species *P. scoparia* and *P. communis* [12] as well as in sweet cherry (*P. avium*) [1]. However, the effect of H_2O_2 on the main non-enzymatic antioxidants glutathione and ascorbate and on the hormone profile in peach (*P. persica*) seedlings has not been previously explored. Achieving a rapid and uniform seed germination and also obtaining vigorous seedlings are key goals for peach breeding programs [2]. In this work, we used the peach cv. GF305, which is commonly used in breeding programs due to its susceptibility to numerous viruses [2]. H_2O_2 imbibition following cold stratification of GF305 was applied in order to increase the germination rate and reduce the stratification time. The levels of ascorbate, glutathione, ABA, 1-aminocyclopropane carboxylic acid (ACC), indol acetic acid (IAA), jasmonic acid (JA), salicylic acid (SA), zeatin-riboside (ZR), and zeatin (Z) were analyzed in the seedlings in order to associate changes in these variables with enhanced germination and seedling growth.

2. Materials and Methods

GF305 seeds were obtained from Pépinières Lafond (Valréas Cedex, France). Seeds (approximately 500) were treated with a 2% tetramethylthiuram disulfide (TMTD) fungicide solution for 30 min and then incubated for 3 days in distilled water at 25 °C in the dark, with the water renewed daily. Then, the seeds were introduced in mesh bags and placed in plastic trays with vermiculite previously moistened in a cold chamber at 5 °C in order to fulfill vernalization requirements. After 4, 6, and 8 weeks of stratification, the endocarp of 50% of the peach seeds was manually removed. Three batches of seeds with endocarp (+ endo) and three without endocarp (– endo) were treated as follows: seeds without imbibition (C); seeds imbibed in distilled water (Im); and seeds imbibed in 10 mM H_2O_2 (Im H_2O_2). For seeds without endocarp, the imbibition lasted for 24 h, whereas for seeds with endocarp, the imbibition lasted for 48 h. Afterwards, the seeds were sowed in 48-cell trays containing peat substrate and incubated in a growth chamber at 25 °C, 70% relative humidity, and 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ white light with a 16/8 h photoperiod (light/dark) for 14 days. Finally, seedlings were divided into shoots and roots and weighed to register the fresh weight (FW). The samples were then snap-frozen in liquid nitrogen and stored at –80 °C for further analyses.

The non-enzymatic antioxidants ascorbate and glutathione were determined as previously described [13–15]. Briefly, samples were homogenized in 1 M $HClO_4$, then centrifuged at 12,000× *g* for 10 min and the pH supernatant was adjusted to 5.5–6 with 5 M K_2CO_3 . Then, reduced (GSH) and oxidized (GSSG) glutathione were analyzed using dithio-bis-2-nitrobenzoic acid and glutathione reductase in the presence of NADPH at 412 nm [13–15], whereas reduced ascorbate (ASC) was measured by recording the absorption at 265 nm, and the total ascorbate was determined via oxidation to non-absorbing oxidized ascorbate (DHA) in the presence of ascorbate oxidase [13–15]. Hormones (abscisic acid (ABA), 1-aminocyclopropane carboxylic acid (ACC), indol acetic acid (IAA), zeatin (Z), zeatin-riboside (ZR), salicylic acid (SA), and jasmonic acid (JA)) were extracted from plant tissues and analyzed using a high-performance liquid chromatography/mass spectrometry (HPLC/MS) system consisting of an Agilent 1100 Series HPLC (Agilent Technologies,

Santa Clara, CA, USA) connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies, Santa Clara, CA, USA) following previously published methodology [3]. In order to reduce analysis costs and taking into account that no differences were observed between C and Im seeds in terms of germination rate and seedling development, the determination of non-enzymatic antioxidants and hormones levels were carried out by comparing the seeds submitted to imbibition (Im vs. ImH₂O₂).

The experiments were repeated twice with similar results. Analyses for germination and FW measurements were done on the data of 20–40 specimens, whereas analyses for antioxidants and hormones contents were done on at least three biological replicates, each one based on the pull of shoots or roots of 10 specimens. The data were analyzed by one- or two-way ANOVA using SPSS 22 (IBM Corp., Armonk, NY, USA) software, followed by Duncan's multiple range test ($p \leq 0.05$) in the case of data of germination percentage and seedling FW.

3. Results and Discussion

Cold wet stratification has been widely used for the germination of seeds from *Prunus* species. In this sense, 12 weeks of stratification has been proven to fulfill vernalization requirements in *Prunus*, leading to dormancy breaking and germination percentages near 95% [1,2], whereas decreasing stratification time to 8 weeks reduced germination percentage up to 40% [1]. In this work, we attempted to reduce the stratification time by using H₂O₂ imbibition after stratification. Four weeks of stratification resulted in a very low germination rate (below 10%) with no significant differences among treatments (data not shown). Six weeks of stratification led to germination percentages below 50% in all cases (Figure 1). In this sense, although seeds without endocarp showed significantly higher germination rates than seeds with endocarp, no significant differences among imbibition treatments were found (Figure 1). On the other hand, in 8 weeks-stratified seeds, the imbibition in H₂O₂ remarkably increased the percentage of seeds germination without endocarp up to 86%, compared to non-imbibed seeds (53% germination) and water-imbibed seeds (55% germination; Figure 1). However, the seeds with endocarp showed a lower germination rate (both imbibed and non-imbibed seeds), with the values being statistically comparable to those of 6 weeks-stratified seeds without endocarp (Figure 1). This inhibitory effect of the endocarp on peach seed germination was previously described in peach and could be due to a water uptake delay and the presence of germination inhibitors, such as ABA [2]. According to these results, the subsequent analyses were carried out on seedlings obtained from seeds subjected to 8 weeks of stratification followed by removal of endocarp.

Regarding the seedling growth, in 8 weeks-stratified seeds, the imbibition with H₂O₂ had no effect on it, whereas the imbibition with water after stratification slightly decrease the FW of seedling roots (Figure 2). Thus, after 8 weeks of stratification, H₂O₂-imbibed seeds showed good development and vigor. In comparison to our results, it was previously described that after 12 and 13 weeks of stratification, the resulting plants displayed good development, with no differences between seeds with or without endocarp, whereas a negative effect on seedling growth was observed when a longer period of stratification was applied [2]. Moreover, different authors have pointed out that stratification periods between 10 and 13 weeks were appropriate for peach cultivars [2]. In seeds from wild almond species, the combined treatment of cold stratification with H₂O₂ and GA₃ reduced the time for germination and increased the germination rate, although a synergistic effect was not found [16]. According to these results and our own results, H₂O₂ appears to be an economic and effective agent for large-scale application in seed germination in *Prunus*.

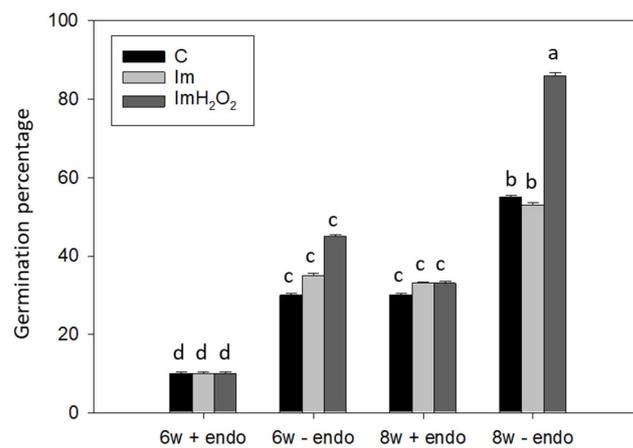


Figure 1. Effect of H₂O₂ imbibition on the germination percentage (%) of peach seeds after 6 (6w) and 8 weeks (8w) of stratification at 5 °C. After stratification, the endocarp of 50% of the peach seeds were manually removed. Three batches of seeds with endocarp (+ endo) or without endocarp (– endo) were treated as follows: seeds without imbibition (C), placed directly from the stratification to the growing trays; seeds imbibed in water (Im); and seeds imbibed in 10 mM H₂O₂ (ImH₂O₂). Seeds were then sowed in trays and incubated at 25 °C with a 16/8-h photoperiod (light/dark) for 14 days. Different letters indicate statistical significance among treatments according to Duncan’s test ($p \leq 0.05$).

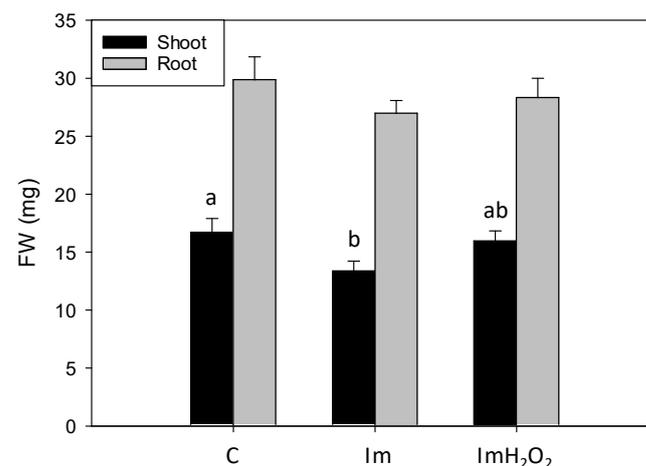


Figure 2. Effect of H₂O₂ treatment on the growth (measured as fresh weight, FW) of peach seedlings resulting from seeds subjected to 8 weeks of stratification followed by endocarp removal. Data represent the mean \pm SE of at least 20 repetitions. Different letters indicate statistical significance among treatments according to Duncan’s test ($p \leq 0.05$).

Seed dormancy is an evolutionary adaptation present in seeds of all temperate fruit species, including peach, that allows seed germination in a favorable season adequate for seedling growth [2]. The presence of a seed coat in stone fruits seeds negatively affects germination, as it constitutes a physical barrier and also contains high levels of ABA [1,2,17]. On the other hand, it has been previously described that H₂O₂ imbibition stimulates seed germination in both dormant and non-dormant seeds, in a manner dependent on the species, as well as the concentration and the timing of application [1–4,6,10,18]. In *P. scoparia*, the combination of cold stratification and 0.5% H₂O₂ was more effective at breaking dormancy than the widely used phytohormone gibberellic acid [12]. This stimulation has been often associated with changes in antioxidative metabolism. In this sense, we observed that in shoots of peach seedlings, H₂O₂ imbibition resulted in a decrease in reduced glutathione (GSH) content, although the glutathione redox state was not affected

because the oxidized form (GSSG) also showed a slight decrease (Table 1). In pea seeds, enhanced seedling growth by 20 mM H₂O₂ and 0.25 mM KNO₃ treatments was also correlated with decreased GSH and GSSG levels [3,19]. However, in seedlings roots, an increase in GSH leading to a higher glutathione redox state was recorded following H₂O₂ imbibition (Table 1). Regarding ascorbate levels, H₂O₂ imbibition produced an increase in both reduced ascorbate (ASC) and oxidized ascorbate (DHA) in seedlings shoots, although the differences were not statistically significant (Table 1). In pea seeds treated with different H₂O₂ concentrations, enhanced seedling vigor was correlated with changes in the levels of enzymatic and non-enzymatic antioxidants [3]. The authors observed that H₂O₂ imbibition led to a slight decline in the glutathione and ascorbate redox state due to a GSH decrease and a DHA increase, respectively. Moreover, a rise in ascorbate peroxidase (APX) activity was also recorded in pea seedlings [3]. Similarly, in peach seedling shoots, we observed a decrease in GSH as well as an increase in DHA (Table 1). In this sense, it has been suggested that ascorbate plays a crucial role during seed germination via stimulation of ascorbate biosynthesis and APX activity, although the possibility that they are the consequence rather than the cause of seed vigor cannot be ruled out [20].

Table 1. Effect of water and H₂O₂ imbibition on the ascorbate and glutathione concentrations in the shoots and roots of peach seedlings resulting from seeds submitted to 8 weeks of stratification followed by endocarp removal. The table displays data for reduced and oxidized glutathione (GSH and GSSG, respectively), glutathione redox state (GSH/(GSH+GSSG)), and reduced and oxidized ascorbate (ASC and DHA, respectively).

	GSH nmol ⁻¹ FW	GSS Gnmol ⁻¹ FW	GSH/ (GSH+GSSG)	ASC nmol ⁻¹ FW	DHA nmol ⁻¹ FW
IM_SHOOT	274.2 ± 13.7	13.1 ± 0.9	0.95 ± 0.01	884.4 ± 62.1	116.6 ± 26.1
IMH ₂ O ₂ _SHOOT	211.5 ± 6.9 *	12.2 ± 0.4	0.94 ± 0.00	1184.6 ± 67.0	162.8 ± 39.0
IM_ROOT	105.7 ± 7.4	12.1 ± 0.5	0.89 ± 0.01	nd	nd
IMH ₂ O ₂ _ROOT	148.6 ± 4.9 *	12.2 ± 0.4	0.92 ± 0.00 *	nd	nd

Data represent the mean ± SE of at least three repetitions. The "*" symbol indicates statistical significance between treatments for either shoots or roots ($p \leq 0.05$). nd: non-detected.

The germination process is linked to important changes in the redox state of the seeds, and a relationship between ROS and plant hormones in this process is well known [21]. It has been widely described that ROS interact in a complex manner with phytohormone networks, triggering signaling pathways that regulate many physiological processes in plants, including seed germination and seedling establishment [4]. In this work, we analyzed the ABA, ACC, IAA, SA, and JA levels and the ratio Z/ZR in shoots and roots of peach seedlings resulting from seeds submitted to 8 weeks of stratification and manual endocarp removal. Seed imbibition with H₂O₂ produced a decrease in ABA and JA in seedling roots. Regarding the rest of the phytohormones, no significant differences were recorded following the H₂O₂ imbibition (Figure 3).

In pea seedlings, H₂O₂ treatment decreased the ABA, IAA, ZR, SA, and JA levels [3]. A decrease in ABA has been traditionally associated with successful seed germination [4,7], with H₂O₂ treatments resulting in a drop in ABA levels [4,10,22], similarly to the one observed in the peach seedling roots (Figure 3). Regarding JA, opposite results have been reported, with either JA inhibiting or promoting the germination process; therefore, the role of JA acid in seed germination is far from being totally understood [4]. Recently, it has been suggested that JA and ABA act synergistically in most of the biological processes, including seed germination [23,24]. A H₂O₂-mediated decrease in ABA and JA levels, such as the one described in pea seeds and seedlings [3,10] as well as peach seedlings (Figure 3), seems to be necessary for seedling growth. In fact, the inhibitory effect of ABA on seed germination in rice was alleviated by impairing JA biosynthesis, suggesting that ABA stimulates JA biosynthesis to then synergistically inhibit seed germination [25]. In this sense, in pea seeds, imbibition with H₂O₂ and ABA overcame the positive effect on seedling growth achieved

by H_2O_2 alone in terms of seedling development, which correlated with a decline in the endogenous H_2O_2 level [26].

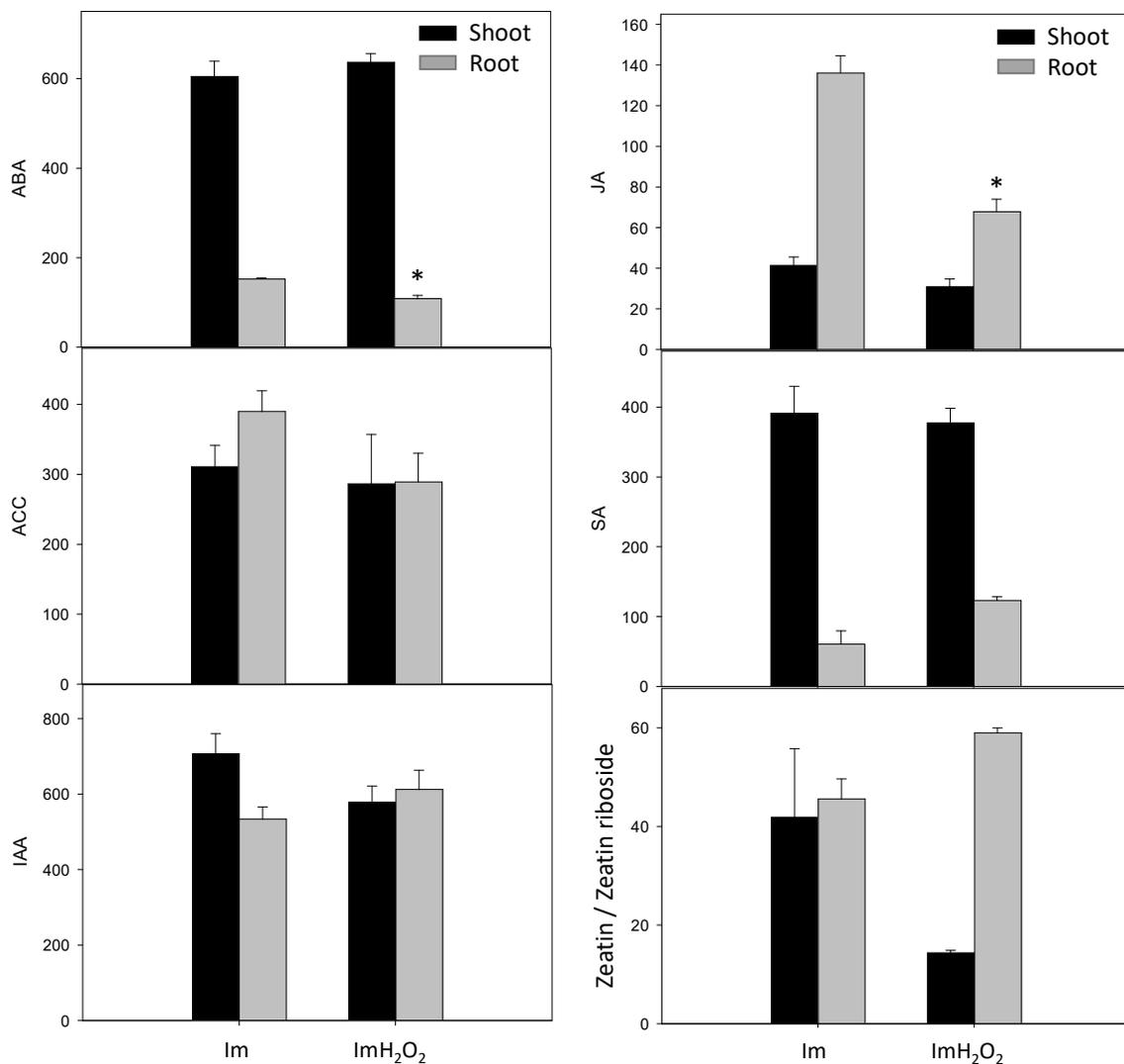


Figure 3. Effect of water and H_2O_2 imbibition on the hormone profile in the shoots and roots of peach seedlings resulting from seeds subjected to 8 weeks of stratification followed by endocarp removal. Data are expressed as $nmol\ g^{-1}\ FW$. Data represent the mean $\pm SE$ of at least three repetitions. The symbol “*” indicates statistical significance between treatments for either shoots or roots ($p \leq 0.05$).

It has been suggested that keeping the Z/ZR ratio towards the active form (Z) could be important for seedling establishment [3], in a process in which ROS are likely involved in the homeostatic regulation of Z and ZR levels [27]. In this study, the Z/ZR ratio increased in root samples and decreased in shoot samples upon H_2O_2 treatment, although significant differences were not found (Figure 3). In addition to its role in the induction of pathogenesis-related proteins and systemic acquired resistance, the role of SA as a developmental regulator is well reported [28]; however, no significant differences among treatments were found under our experimental conditions. In spite of the well-reported role of ethylene in seed germination and seedling development [4,29], no significant differences were observed in the ethylene precursor ACC (Figure 3), as has also been observed in pea seedlings [3]. However, in soybean, it has been suggested that ROS-induced ethylene production during germination stimulates cell elongation in the root tip [29].

4. Conclusions

In this paper, we have described a method for an efficient and unexpensive reduction of the stratification time required for the germination of peach cv. GF305. After a cold stratification period of 8 weeks, endocarp was removed and seeds were imbibed in 10 mM H₂O₂, resulting in seedlings that displayed good development. Compared to non-treated seeds, for which a stratification period of 12 weeks has been established, we reduced the stratification time by 4 weeks. Moreover, stimulation of seedling growth was also achieved, which correlated with changes in non-enzymatic antioxidants and ABA and JA contents. In general, our findings may have practical application on peach breeding programs and nurseries, as well as on other *Prunus* species.

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