Plant Physiology and Biochemistry 94 (2015) 165–173

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Effects of moist cold stratification on germination, plant growth regulators, metabolites and embryo ultrastructure in seeds of *Acer morrisonense* (Sapindaceae)

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ARTICLE INFO

Article history:
Received 20 November 2014
Received in revised form
2 June 2015
Accepted 3 June 2015
Available online 5 June 2015

Keywords:
Acer
Amino acids
Cold stratification
Embryo ultrastructure
Plant growth regulators
Seed dormancy
Seed germination
Sugars

ABSTRACT

Breaking of seed dormancy by moist cold stratification involves complex interactions in cells. To assess the effect of moist cold stratification on dormancy break in seeds of *Acer morrisonense*, we monitored percentages and rates of germination and changes in plant growth regulators, sugars, amino acids and embryo ultrastructure after various periods of cold stratification. Fresh seeds incubated at 25/15 °C for 24 weeks germinated to 61%, while those cold stratified at 5 °C for 12 weeks germinated to 87% in 1 week. Neither exogenous GA_3 nor GA_4 pretreatment significantly increased final seed germination percentage. Total ABA content of seeds cold stratified for 12 weeks was reduced about 3.3-fold, to a concentration similar to that in germinated seeds (radicle emergence). Endogenous GA_3 and GA_7 were detected in 8-week and 12-week cold stratified seeds but not in fresh seeds. Numerous protein and lipid bodies were present in the plumule, first true leaves and cotyledons of fresh seeds. Protein and lipid bodies decreased greatly during cold stratification, and concentrations of total soluble sugars and amino acids increased. The major non-polar sugars in fresh seeds were sucrose and fructose, but sucrose increased and fructose decreased significantly during cold stratification. The major free amino acids were proline and tryptophan in fresh seeds, and proline increased and tryptophan decreased during cold stratification. Thus, as dormancy break occurs during cold stratification seeds of *A. morrisonense* undergo changes in plant growth regulators, proteins, lipids, sugars, amino acids and cell ultrastructure.

1. Introduction

Cold stratification is commonly used to break dormancy in water-permeable seeds of many temperate-zone species, especially those whose seeds germinate in the natural habitat in spring. In this dormancy-breaking treatment, seeds are incubated on a moist substrate at low (0–10 °C) temperatures. For many species, 5 °C is optimal for dormancy break (Baskin and Baskin, 2014), but in some species temperatures lower than 5 °C are more effective than 5 °C. For example, 0 °C was more effective than 5 °C in overcoming physiological dormancy after the embryo in seeds of *Aegopodium podagraria* had become fully developed (Phartyal et al., 2009). In some species, cold stratification is not very effective in breaking dormancy unless it is preceded by several weeks of warm (≥15 °C) moist stratification, e.g. *Taxus* species (Chien et al., 1998), *Cephalotaxus* (Yang et al., 2011) and some *Fraxinus* (Bonner and Karrfalt, 2008) and *Viburnum* (Chien et al., 2011) species. As dormancy break occurs via cold stratification, germination percentages and rates increase, and often the temperature range over which seeds will germinate increases (Baskin and Baskin, 2014). Also, many biochemical and structural changes are known to occur in seeds during cold stratification (Bewley et al., 2013).

During dormancy-break, the level of abscisic acid (ABA) is high in freshly matured seeds and decreases with warm or cold stratification, and levels of GA_1, GA_3, GA_4 or GA_7 (gibberellins) increase after cold stratification (Chen et al., 2008, 2010; Lewak, 2011). The

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http://dx.doi.org/10.1016/j.plaphy.2015.06.004
0981-9428/

effects of cold stratification on enzymatic activities and reserves in seeds of woody plants have been investigated. Cold stratification (1) broke embryo dormancy of *Malus domestica* seeds via catabolism of lipids, sugars and proteins by hydrolytic or proteolytic enzymes (Lewak, 2011); (2) promoted germination of *Juglans regia* seeds by activating hydrolases that catabolized proteins and thus increased the quantity of amino acids (Einali and Sadeghipour, 2007); and (3) broke dormancy in seeds of *Corylus avellana* and increased starch content, probably as a result of gluconeogenesis from products of reserve lipid hydrolysis (Li and Ross, 1990).

Ultrastructural studies of embryo cells have revealed that there is gradual mobilization of lipid and protein bodies in cold stratified seeds. Cold stratification of *M. domestica* seeds for 90 days not only broke dormancy but caused degradation of protein bodies and lipids, and the degraded lipids supposedly were converted into starch (Dawidowicz-Grzegorzewska, 1989). Warm stratification of yew (*Taxus mairei*) seeds for 6 months decreased the number of lipid and protein bodies and increased the number of mitochondria, plastids, dictyosomes, vacuoles and microbodies in the shoot apical meristem, and then cold stratification for 3 months initiated cell division and finally germination (Chien et al., 1998). Cold stratification of aged *Picea mariana* seeds resulted in repair of ultrastructural damage in the seeds and increased germination percentages (Wang and Berjak, 2000).

Although detailed biochemical and structural studies have been conducted on seeds of various species during cold stratification, our knowledge is deficient in what happens during cold stratification of seeds of some of the major genera of trees. One such genus is *Acer*. This genus consists of about 148 species of deciduous (rarely evergreen) trees and shrubs distributed in the northern hemisphere, including Europe, North America and Asia, which is the geographic center of the genus (Zasada and Strong, 2008). There are about 140 species in China, including six species in Taiwan (Woody Flora of China, 2004). Many species of *Acer* have ornamental value and thus are widely used for landscaping. Fruits (samaras) of most *Acer* species are dormant and require cold stratification or sometimes warm plus cold stratification for dormancy to be broken (Baskin and Baskin, 2014). The length of the cold stratification period required to break dormancy in seeds of *Acer* ranges from c. 45 to 90 up to 180 days, depending on the species (Baskin and Baskin, 2014).

Pinfield and Gwarazimba (1990) found that the ABA content of the embryo in fresh *Acer* seeds was high but decreased with warm and/or cold stratification. The decrease in ABA content, probably in conjunction with weakening of the testa and pericarp, promoted rapid seed germination. The purpose of our research was (1) to determine the effect of cold stratification and exogenous GA_3 and GA_4 on germination, and (2) monitor changes in endogenous levels of ABA, GA, metabolites and embryo ultrastructure during cold stratification of seeds of *Acer morrisonense*. This species is endemic to Taiwan, and it is a dominant tree in forests at elevations of 1800m–2500 m (Li and Lo, 1993; pers. observ.).

2. Materials and methods

2.1. Fruit collection

Freshly matured samaras of *A. morrisonense* were collected from Tsuifeng at an elevation of 2400 m, Nanto County, Central Taiwan (24°06′20″N, 121°11′13″E) during early November 2011 and late December 2014. All studies, except the experiment on effects of exogenous GA_3 and GA_4 on germination were conducted using seeds collected in 2011. The GA experiment was done with seeds collected in 2014. After air-drying at room temperatures (21–23 °C) for 1 week, the wing was removed by hand from each fruit, which were stored for 1 week at 5 °C before use. De-winged fruits (hereafter seeds) were used in all studies. Three hundred dry seeds were frozen in liquid nitrogen and stored at −80 °C to await analyses. Seeds (n = 50) were 5.13 ± 0.52 mm long, 3.51 ± 0.23 mm wide and 2.39 ± 0.42 mm thick, and there were 26,800 seeds per liter and 77,750 seeds per kg. Moisture content of fresh seeds was 15.4 ± 0.3% as determined by oven drying for 17 h at 103 °C (International Seed Testing Association, 2007).

2.2. Effect of temperature and cold stratification on germination

To determine the temperature requirements for germination, fresh seeds were mixed with moist sphagnum moss (cut into small pieces) at a 75–80% moisture content in sealed polyethylene (PE) bags (0.04 mm thick). Seeds were incubated for 36 weeks at 30/20, 30/15, 25/15, 20/10, 15/5 °C and 25 °C. At the alternating temperature regimes, the high and low temperature was given for 12 h each day. Seeds were exposed to light 12 h each day and for those incubated at the alternating temperature regimes during the high-temperature phase of the daily cycle (hereafter light). The light source was white fluorescent tubes, and photon irradiance (400–700 nm) was about 60–80 μmol m^{-2} s^{-1}. The moist sphagnum provided a good germination medium and prevented the spread of mold because it contains the fungus *Trichoderma* and actinomycetes that are antagonistic to microorganisms (Wang et al., 1998). Due to the coarseness of the sphagnum moss, most seeds received some light, but at any given point in time a few of them may have been in darkness. However, at weekly intervals the contents of each bag were poured onto a table to facilitate examination of seeds for germination. After germination was monitored, nongerminated seeds and sphagnum moss were returned to the bag, resulting in a re-shuffling of seeds with regard to their position in/on the sphagnum moss and thus the light they received. Consequently, all seeds were in light part (or all) of the time in the incubator. Each treatment consisted of three replications of 50 seeds each. At 1-week intervals, seeds with a radicle ≥2 mm in length were recorded as germinated and removed from the bag. Results are expressed as mean (±1 SE) germination percentage or as germination speed, i.e. time to reach 50% germination of viable seeds (t_{50}) (Bewley et al., 2013).

To determine the response of seeds to cold stratification, fresh seeds were mixed with moist sphagnum moss (as described above) and placed in darkness for 0 (control), 4, 8, 12 and 16 weeks. After each treatment, seeds were incubated for 12 weeks in light at 25/15 °C as described above. The control was fresh seeds incubated at 25/15 °C throughout the experiment. Three hundred seeds each cold-stratified at 5 °C for 4, 8 and 12 weeks and 200 seeds with radicles emerged were immediately frozen in liquid nitrogen and stored at −80 °C until further analyses could be performed.

2.3. Effect of exogenous GA_3 and GA_4 on germination

To promote infiltration of GA_3 or of GA_4 solutions through the pericarp and seed coat and into the embryo, seeds collected in December 2014 were placed in 0, 25, 250 and 2500 μM solutions of GA_3 (potassium salt, 95% purity, Sigma, St Louis, Missouri, USA) or GA_4 (90% purity, from Professor Lewis N. Mander, Australian National University), which were then immediately placed in a vacuum container at a pressure of 30 cmHg (0.4 atm) for 16 h. Vacuum infiltration previously has been used in testing the effects of GA_3 and of GA_4 on seed germination (Loveys and Jusaitis, 1994; Chen et al., 2007). The treated seeds were mixed with moist sphagnum moss and incubated at 25/15 °C for 56 days. Also, seeds treated with 2500 μM GA_4 were incubated at 25/15 °C for 16 days and then