



Efficient in vitro germination of the endangered Sinai hawthorn (*Crataegus sinaica*)

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Received: 24 August 2024 / Accepted: 6 February 2025 / Published online: 26 February 2025
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Abstract

Sinai hawthorn, *Crataegus sinaica*, is a rare wild tree species of economic importance with potential for medicinal uses. The in vivo seed germination of this tree species is extremely low. The present study aimed to develop an efficient in vitro seed germination that can contribute to the successful propagation, restoration, and preservation of this critically endangered species. The study examined factors that could enhance seed germination frequency (GP) and dormancy break of mature seeds. This included stratification temperature, cytokinin type and concentration, sinapic acid (SA) concentration, gibberellic acid (GA), and substituting nutrient media with water. Cold-stratified seeds for 4 weeks at 7 °C showed highest GP of 29.3%. GP of 36% was obtained on media containing 0.5 mg/L of each 6-Benzyladenine (BA) and meta-Topolin (mTp). Low concentrations of SA increased GP (50%) in both full and ½ Murashige and Skoog basal media with Gamborg B5 vitamins (MSB5). Sterile ddH₂O supplemented with selected combined GA, SA, and mTp treatment showed a higher GP (46%) compared to the control. The study investigated the physiology of seed maturation during late growing season and after stratification and their correlation with germination efficiency. Stratification at 7 °C increased total soluble sugars, decreased abscisic acid (ABA) content, and increased GA. This study presents the first report on seed germination in Sinai hawthorn, highlighting the importance of environmental and physiological treatments for improved efficiency. It also offers a simple, inexpensive, method for in vitro seed germination with high efficiency that may be applied in propagation and restoration.

Key message

The study reports successful in vitro germination of the endangered Sinai hawthorn. Seed primary metabolic and hormonal behavior provides key factors for future optimization of germination, micropropagation, and species conservation.

Keywords In vitro germination · Cold stratification · Plant growth regulators · Seed dormancy · Micropropagation · Conservation biology

Introduction

Hawthorn, *Crataegus x sinaica* Boiss. (*C. sinaica*), is a critically endangered medicinal plant with potential therapeutic and pharmacological effects, including the potential for improving heart-related and hepatic disorders (Refaat et al. 2010) and possessing antioxidant, antimicrobial, and

anti-nociceptive properties (Abdel-Rahman et al. 2022; Alsharif et al. 2022).

Sinai hawthorn, native to the Middle East, is primarily found south of the Sinai Peninsula in Egypt, in the Saint Catherine mountains. It faces an extremely high risk of extinction (Shaltout and Bedair 2023). Its distribution is limited due to extremely low seed germination, overgrazing, human disturbance, especially its use as firewood, and due to climatic change (Moustafa et al. 2019). Conservation efforts for desert trees are particularly important because they are vital for plant community establishment, soil stabilization, shade, microhabitats, nutrient cycling, and wildlife habitat in arid environments. Conservation efforts preserve biodiversity, balance ecosystems and support local communities' livelihoods (Robinson 2003; Xie et al. 2023).

Communicated by Adeyemi Oladapo Aremu.

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Seed germination of Sinai hawthorn is low due to both exogenous and endogenous dormancy, caused by a hard, stony endocarp and immature embryos (Göktürk and Yildirim 2020). Seed dormancy is a crucial adaptive strategy in wild higher plants, influencing plant species survival probability. It can be influenced by environmental and genetic factors, and seeds vary in dormancy status and degree (Tognacca and Botto 2021; Klupczyńska and Pawłowski 2021). To improve germination, releasing endogenous embryo dormancy in *Crataegus* species, seeds may be treated with cold or warm stratification. Physical dormancy can be eliminated by removing the hard stony endocarp, and acid scarification pretreatment (Morgenson 2000; Bujarska-Borkowska 2002, 2006, 2007; Ahmadloo et al. 2017; Gokturk et al. 2017; Kheloufi et al. 2019; Göktürk and Yildirim 2020).

The nutritional requirements for *C. sinaica* seed germination and seedling growth in situ and in vitro are not yet identified, but can vary and depend on factors like habitat and life history (Diantina et al. 2020).

Seed reserves affect germination differently depending on the species and reserve amount. *Tilia miqeliiana* seeds showed decreased protein and fat levels during dormancy release (Wu and Shen 2021). Seed contents of soluble sugar and protein were positively correlated with germination rate in six wild grassland plant species (Zhao et al. 2018).

Different environmental factors and endogenous hormones control the induction and break of seed dormancy. Absciscic acid (ABA) and gibberellins (GAs) are known as key regulators of seed dormancy (Soppe and Bentsink 2016). Limited data is reported about the content of ABA in *Crataegus* seeds. ABA levels were elevated during seed dormancy and inhibited seed germination in *C. azarolus* (Qrunfleh 1991), *C. pinnatifida*, and *C. sanguinea* (BingZhen et al. 1998). These levels decreased with stratification, correlating with dormancy release. Other phytohormones mediate the balance between ABA and GA at the level of biogenesis or signaling (Shu et al. 2016). Both GA and cytokinin were found to promote dormancy break by antagonizing ABA-mediated inhibitory effects. Cytokinins stimulated seed germination by inhibiting *ABI5* transcription (Wang et al. 2011) or by promoting *ABI5* protein degradation (Guan et al. 2014). In seeds, endogenous cytokinin content has been increased prior to radicle emergence (Van Staden et al. 2010). Cytokinin-containing media promote seed germination in vitro (Nikolić et al. 2006). Topolins, aromatic compounds with cytokinin activities, are used effectively in plant tissue culture as an alternative cytokinin (van der Westhuizen 2014; Ahmad and Strnad 2021). Stratification combined with benzyladenine treatment enhanced seed germination (Sharifi and Pouresmael 2006).

Sinapic acid (3,5-dimethoxy-4-hydroxycinnamic acid) is a naturally occurring bioactive phenolic acid, present in

both free and ester forms. It is a member of the hydroxycinnamic acid family that is found in edible plants, mainly the Brassicaceae species (Chen 2016; Bi et al. 2017; Deng et al. 2021). Sinapic acid (SA) significantly impacts seed development and dormancy regulation in plants, inhibiting ABA-mediated seed germination in *Arabidopsis* lines (Bi et al. 2017) by promoting the conversion of ABA to its inactive forms and enhancing the expression of glucosyltransferases involved in ABA metabolism, leading to lower free ABA and higher ABA-glucose esters. The accumulation of SA esters is crucial for modulating responses to ABA in *Arabidopsis*. Mutants such as *sng2* (deficient in sinapoylcholine synthesis) and *btr1* (defective in sinapoylglucose production) showed reduced germination rates under ABA treatment. In contrast, the *sng1* mutant which accumulates sinapoylglucose was less sensitive (Bi et al. 2017). SA negatively impacts seed dormancy in *Davidia involucrata* (Deng et al. 2021). Significant levels of sinapic acid accumulated in seeds that underwent 12 months of moist sand stratification or longer. Therefore, increasing the levels of this compound may enhance germination.

The present research aimed at enhancing seed germination of the recalcitrant *C. sinaica* using in vitro culture methods and at understanding specific factors contributing to efficient germination. Studying the factors that promote successful dormancy break and seed germination, including cold stratification, cytokinin concentration, sinapic acid concentration, and long-term cold incubation were carried out. Changes in endogenous hormones and primary metabolites during late seed maturation and subsequent germination were examined. Elucidating the behavior of the studied metabolic and hormonal factors will positively contribute to the successful, efficient and reproducible seed germination. Micropropagation, conservation and restoration of the species will be feasible by developing strategies and techniques to improve germination rates and strong seedling growth.

Material and methods

In vitro germination and dormancy breaking

Plant material

Fully ripened fruits were collected from two locations in Wadi Gebal in the Saint Catherine Protectorate (South Sinai, Egypt) in October 2019. Localities and GPS data are shown in Fig. 1. The plant samples were identified by Dr. Loutfy Mohsen, Professor of Plant Taxonomy, Botany Department, Faculty of Science, Helwan University, Egypt. Samples were collected under guidelines and with permission from the Saint Catherine Protectorate.

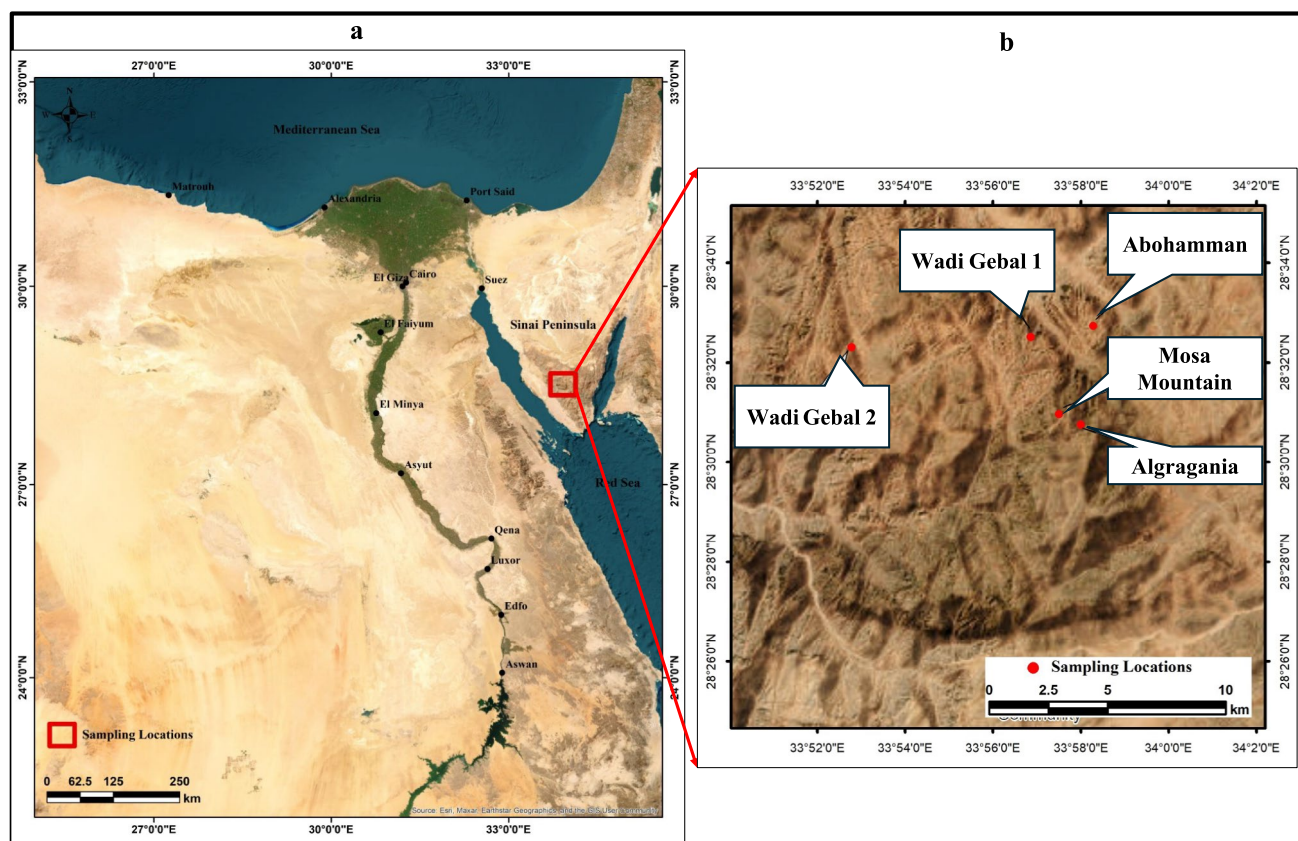


Fig. 1 Location map of the study area in the Sinai Peninsula, Egypt. **a** Study locations in the Saint Catherine Protectorate, South Sinai; **b** Sampling locations for *C. sinaica* to test in vitro germination and dormancy breaking of seeds: Wadi Gebal 1 (28.54026° N, 33.87907° E, 1809 m), Wadi Gebal 2 (28.52031° N, 33.95973° E, 2140 m).

Herbarium specimen with a voucher number (010563) was deposited at the herbarium of the Faculty of Science, Helwan University (HEU).

Seeds were extracted by removing the fleshy pericarp and washed thoroughly. Seeds with stony endocarp were used for cold stratification treatments. The stony endocarp was removed after the stratification treatment and before seed sterilization for all experiments (Fig. 1 Supplementary data).

Seed sterilization

Seeds were washed under tap water for 30 min., followed by soaking in 95% ethanol for 1 min., immersed in fungicide (Rezolex 1 g/L) for 10 min. and washed in ddH₂O for 10 min. Surface sterilization with calcium hypochlorite was for 20 min. Under aseptic conditions, seeds were rinsed 3 times in distilled water (10 min. each) and moved onto sterile filter paper until cultured (Fig. 2 Supplementary data).

Sampling locations to test phytochemical and hormonal changes in seeds during maturity and germination: Abohamman (28°32'36.2" N, 33°56'58.7" E, 1911 m), Mosa Mountain (28°32'49.4" N, 33°58'22.8" E, 2023 m), and Algragania (28°30'44.8" N, 33°58'09.2" E, 2160 m)

Effect of cold stratification temperatures and incubation periods

Two cold temperature treatments were tested: (i) 7 °C and (ii) – 20 °C. After extracting seeds from fruits, seeds were divided into 8 groups for the stratification treatment. Four groups were stored at 7 °C, while the other four groups were stored at – 20 °C. Seeds were then collected for in vitro germination after 2, 4, 6, and 8 weeks, respectively, from each group. The stratified and non-stratified (control) sterile seeds were cultured on a modified Murashige and Skoog (1962) basal medium with Gamborg B5 vitamins (Gamborg et al. 1968) (MSB5). The medium (Phytotechnology Laboratories) and supplemented with 0.5 mg/L BA, 0.1 mg/L mTp, 0.1 g/L myo inositol, 20 g/L sucrose. Media was gelled using 1.5 g/L phytigel (Sigma Aldrich). Three replicates with 16 seeds each were cultured.

Effect of different concentrations of 6-Benzyladenine (BA) and meta-Topolin (mTp)

To test the effect of 2 cytokinin types (BA and mTp) and their different concentrations on germination frequency, seeds stratified at 7 °C for 4 weeks were used. Sterile seeds were aseptically transferred to MSB5 medium supplemented with a combination of different concentrations of BA (0.5, 1.0, 2.0 mg/L) with 0.5, 1.0, 2.0 mg/L mTp. All media were supplemented with 0.1 mg/L NAA, 15 g/L sucrose, and 15 g/L glucose. Media were gelled using 1.5 g/L phytagel (Sigma Aldrich). Three replicates were carried out (12 seeds/replication).

Effect of different concentrations of sinapic acid (SA) and media strength

Stratified seeds (at 7 °C for 4 weeks) were sterilized and aseptically cultured onto: (1) full-strength MSB5, (2) half-strength MSB5. Each medium strength treatment was supplemented with different concentrations of SA (0.0, 1.0, 2.0, 3.0) mg/L. The media was gelled using 5.5 g/L agar (Sigma Aldrich). Three replicates were carried out (12 seeds/replication).

All media pH was adjusted to 5.8 and autoclaved at 120 °C for 20 min. All media were supplemented with 0.1 g/L myo-inositol and 20 g/L sucrose.

All cultures were incubated for one week in the dark at 25 ± 2 °C, then transferred to a 16/8 h. (light/dark) photoperiod using white, fluorescent light (350–500 lx).

Effect of growth regulators supplemented in sterile ddH₂O on germination

To initiate a technically feasible, simple and applicable protocol for germination, sterile stratified seeds at 7 °C for 4 weeks, were aseptically transferred to jars containing 50 ml of ddH₂O (in replacement of MSB5 culture medium) and supplemented with either GA₃ (200 mg/L) and SA (1 mg/L), or 0.5 mg/L mTp, 200 mg/L GA₃, 0.5 mg/L BA, 1 mg/L SA. Three replicates (12 seeds/replication) were cultured. Seeds were incubated at 7 °C under dark conditions for all cultures for 8 weeks.

Changes in seed physiology during maturity and germination

Plant material

Fruits from several different genotypes were collected from 3 localities: Abohamman = L1, Mosa Mountain = L2, and Algargania = L3, (Fig. 1), in Saint Catherine Protectorate,

south Sinai, Egypt, during August, September, and October 2019 (collection time/date).

Primary metabolites

Seeds from different genotypes collected from 3 different localities, were extracted by removing the fleshy pericarp and the hard stony endocarp. For seeds stratified at 7 °C the hardy stony endocarp was removed after the cold treatment. Seeds were air dried at room temperature. Seed total soluble sugars, total carbohydrates, total protein, GA₃ and ABA concentrations were measured from 3 different localities during fruit ripening and seed development and maturation in August, September, and October was determined. A sample of seeds collected during October were cold stratified and their primary metabolites and GA₃ and ABA concentrations measured.

Total soluble sugars (TSS)

One hundred milligrams (mg) of dry powdered seeds were mixed with five ml of ethanol 70%. After centrifugation at 4000 rpm for 10 min (min), the supernatant was diluted to 15 ml with distilled water. The anthrone technique as described by Umbreit et al. (1959) was used to determine TSS. Three milliliters sample and 6 ml anthrone solution (2 g/L H₂SO₄ 95%) were mixed and maintained in a boiling water bath for 3 min. and then cooled. The developed color was measured using a spectrophotometer (Jenway 6300) at 620 nm. Measurements were performed in triplicate for each treatment. A standard curve of glucose was used to calculate the concentration of TSS in samples.

Total carbohydrates (TC)

Thirty mg of dry powdered seeds were hydrolyzed in 10 ml of 1N H₂SO₄, in digestion tubes, (80–90 °C) for 8 h. then to 30 ml with distilled water. Then the TC was determined by using anthrone procedure as described by Umbreit et al. (1959)

Total soluble proteins (TSP)

One hundred mg of dry powdered seeds were mixed in 5 ml of 70% ethanol, then centrifuged at 4000 rpm for 20 min. The supernatant was diluted to 15 ml with ddH₂O. The procedure of Lowry et al. (1951) was followed. Two ml plant extract was mixed with 10 ml freshly mixed solution (50:1 v/v) of 2% sodium carbonate in 0.4% sodium hydroxide and 0.5% copper sulphate in 1% sodium tartrate. The mixture was left for 10 min before the addition of 1 ml of Folin. After 30 min, the optical density of the mixture was measured using a Jenway 6300 spectrophotometer at 750 nm. Three

replicates were measured for each treatment. The protein content of samples was determined using a standard curve of bovine serum albumin.

Endogenous phytohormones (ABA, and GA3) analysis

Phytohormones were analyzed at the center of Arid Land Agriculture Research and Services (ALAR), Faculty of Agriculture, Ain Shams University. Fresh seeds (10 g) were collected from Saint Catherine in 2019 (August, September and October). Seeds collected in October were stratified at 7 °C for 4 weeks. Phytohormone extraction was according to a modified method described by Wasfy and Orrin (1975). Samples were ground in cold 80% methanol, the macerated tissues transferred to a flask with fresh methanol, and the volume was adjusted to 20 ml of methanol for 24 h at 0 °C and then vacuum filtered through filter paper Whatman No 42. The residue was returned to the flask with a fresh volume of methanol, stirred for 30 min. and filtered again. The procedure was repeated once more, and the combined extracts were evaporated to the aqueous phase in a rotator flash evaporator. The aqueous phase (10–30 ml) was adjusted to pH 8.6 with 1% NaOH and partitioned 3 times with equal volumes of ethyl acetate. The combined ethyl acetate fraction was evaporated to dryness and held for further purification. The aqueous phase was adjusted to pH 2.8 with 1% HCl and portioned into 3 times equal volumes of ethyl acetate. The remaining aqueous phase was discarded. The combined acidic ethyl acetate volume was reduced (fraction 1), for high-performance liquid chromatography (HPLC) to determine the acidic hormones (ABA and GA3). Injection of 10 µl into HPLC 510 was used for identification and determination of hormones using a data model (Waters 746), detector (Waters 2489 UV/Visible detector), and pump (Waters 1525 Binary HPLC pump). The chromatography was equipped with a 3.9 mm × 300 mm µ Bondapak C18 capillary column. The HPLC was set to run at 25 °C. Standards of GA3, and ABA were used. The identification and characterization of peaks in samples under investigation were achieved using the area of peaks and retention time of different phytohormones of authentic standards.

Statistical analysis

IBM Statistical Product and Service Solutions, SPSS software Version 26 for Windows was used (George and Mallery 2019). All samples were analyzed in triplicate, in a completely randomized design. Mean comparison was determined at $P < 0.05$ using Duncan's Multiple Comparison Test. All results were expressed as mean value $\pm$ standard error (SE).

Results

In vitro germination and dormancy breaking of *C. sinaica*

To test the different factors that may enhance seed germination and dormancy breaking in *C. sinaica*, the effects of cold stratification, cytokinin types and concentrations, sinapic acid, gibberellic acid treatments were studied. The replacement of nutrient media with water as a growth substrate to simplify future application trials in the greenhouse and field to germinate and propagate the species was evaluated.

Effect of two cold stratification temperatures and different incubation periods

Stratification temperature at 7 °C (T1), stratification duration (D), and interaction ($T \times D$) were significantly different at $P < 0.05$, while stratification temperature at -20 °C (T2) did not significantly influence the seed germination percentage, (Table 1). Cold stratification treatment at 7 °C and -20 °C for 4 weeks enhanced the germination and resulted in the highest germination percentages (GP) of 29.3% and 21%, respectively (Fig. 2a; Table 1 Supplementary data). Regardless of the stratification duration, stratification at 7 °C significantly increased the GP by 64% compared to the control (non-stratified seeds) as presented in Fig. 2b. Four-week stratification duration significantly increased the GP to 25.2%, regardless of the stratification temperature (Fig. 3c). Moreover, increasing the stratification duration over 4 weeks decreased germination frequency by about 48% at 6 weeks and 61% at 8 weeks respectively.

Effect of different concentrations of 6-benzyladenine (BA) and meta-Topolin (mTp)

The response of hawthorn seeds to two cytokinin types (BA and mTp) at different concentrations (0.5–2 mg/L) differed significantly at $P 0.05$ (Table 2). Medium supplemented with a combination of BA (0.5 mg/L) and

Table 1 Analysis of variance for the effect of 2 cold stratification temperatures (T1: 7 °C; T2: -20 °C), and different stratification durations (D: 2, 4, 6, and 8 weeks) on the mean germination percentage of *C. sinaica* seeds cultured on MSB5

Source of variation	df	MS	F	$P < 0.05$
Duration (D)	4	114.954	6.524	0.008
Temperature (T)	2	33.730	7.875	0.021
$T \times D$	8	122.544	4.537	0.004
T1 $\times$ D	4	180.725	7.129	0.006
T2 $\times$ D	4	66.767	2.678	0.094

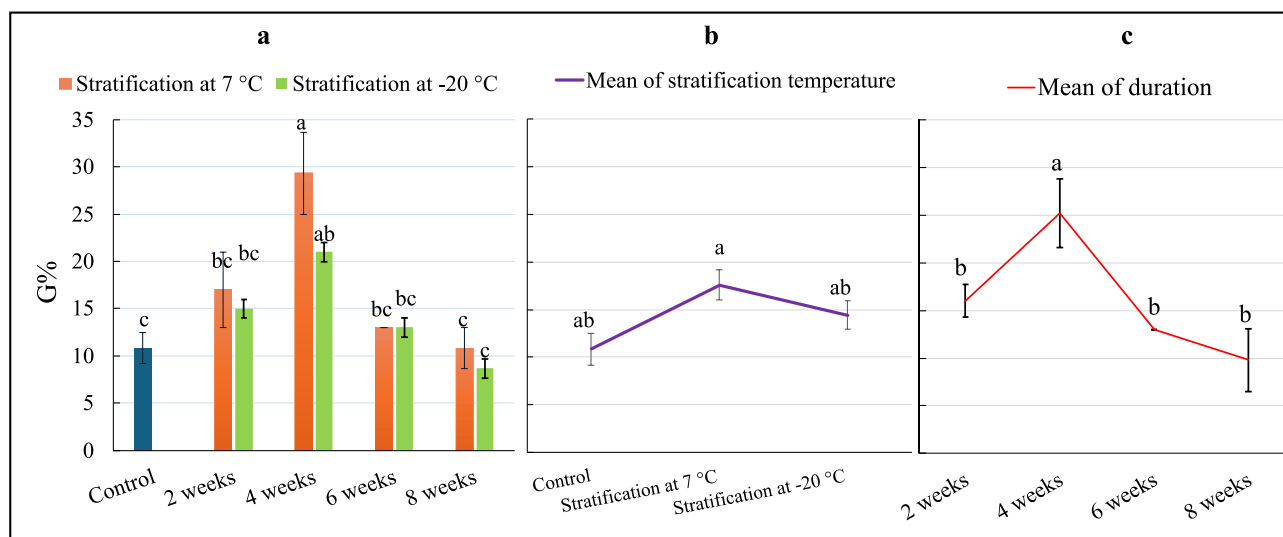


Fig. 2 Germination frequency of *C. sinaica* seeds (mean $\pm$ standard error) as affected by: **a** two stratification temperatures (Control, 7 °C or – 20 °C) for different durations (2, 4, 6, and 8 weeks), **b** means

pooled for each stratification temperature, and **c** means pooled for each stratification duration. Bars with different alphabets indicate significant differences at $P < 0.05$

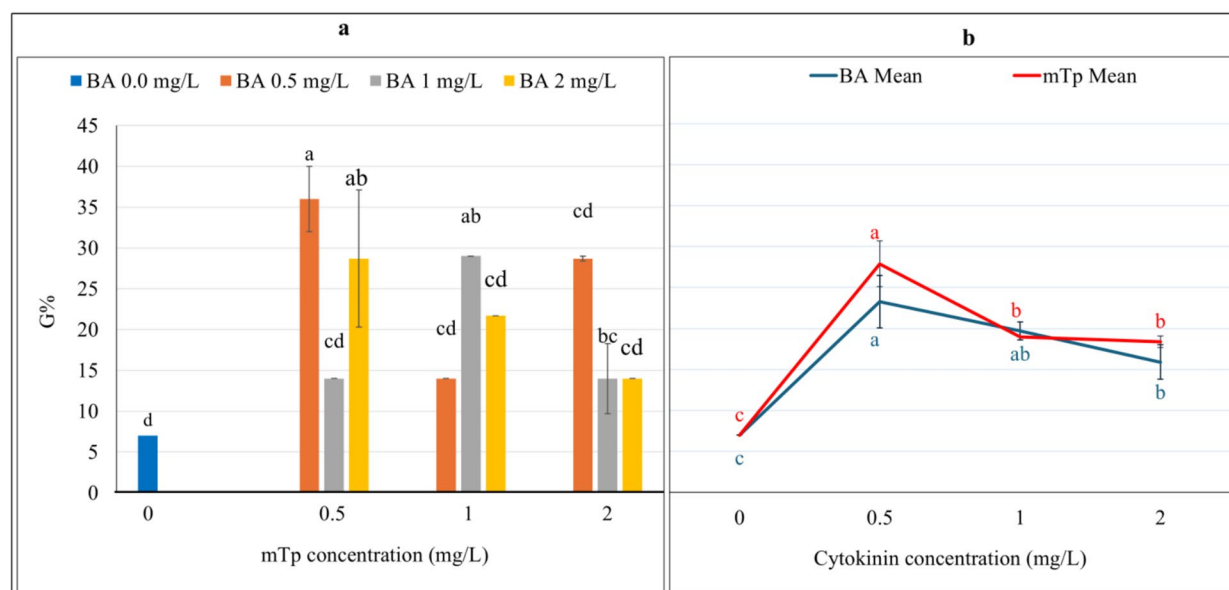


Fig. 3 Germination frequency of in vitro *C. sinaica* germinated seeds as affected by: **a** interaction between two cytokinins (BA and mTp) at different concentrations (0.0, 0.5, 1, and 2 mg/L), **b** concentration

of each cytokinin (BA or mTp). All seeds were stratified at 7 °C for 4 weeks, then cultured on MSB5 media. Different alphabets indicate significant differences at $P < 0.05$

Table 2 Analysis of variance for the effect of 2 cytokinins (BA and mTp) on the mean germination percentage of in vitro cultured *C. sinaica* seeds stratified for 4 weeks at 7 °C

Source of variation	df	MS	F	$P < 0.05$
BA	3	147.311	12.554	0.002
mTp	3	219.915	34.950	0.000
BA $\times$ mTp	9	250.478	7.927	0.000

mTp (0.5 mg/L) significantly gave the highest GP (36%), followed by BA (1.0 mg/L) and mTp (1.0 mg/L) (Fig. 3a; Table 2-a supplementary data). In terms of each cytokinin type and concentration, exogenous BA and mTp at low concentrations enhanced GP more effectively than at higher concentrations. Generally, the effect of mTp treatment on GP was higher than that of BA treatment at the same concentration (Fig. 3b; Table 2-b Supplementary

data). A three-fold increase in GP was observed in the mTp treatment at 0.5 mg/L, while the BA treatment at 0.5 mg/L showed a twofold increase compared to the control treatment. Interaction of both BA and mTp at 0.5 mg/L optimized the germination percentage; the developed seedlings were healthy (Fig. 4c) and acclimatized after 2 months (Fig. 4f).

Effect of different concentrations of sinapic acid and media strength

Media strength, SA concentration, and their interaction were all significant at $P < 0.05$ (Table 3). The highest GP was observed in the full-strength MSB5 media (75%), supplemented with 2 mg/L SA (Fig. 5a; Table 3 Supplementary data). Moreover, full-strength MSB5 media increased the germination percentage by about twice as much as $\frac{1}{2}$ MSB5 (Fig. 5b). Application of SA at low concentrations (1 and 2 mg/L) increased the germination percentage of stratified seeds by about 47%, regardless of the media strength. In contrast, higher concentrations of SA suppressed seed germination in both media (Fig. 5c).

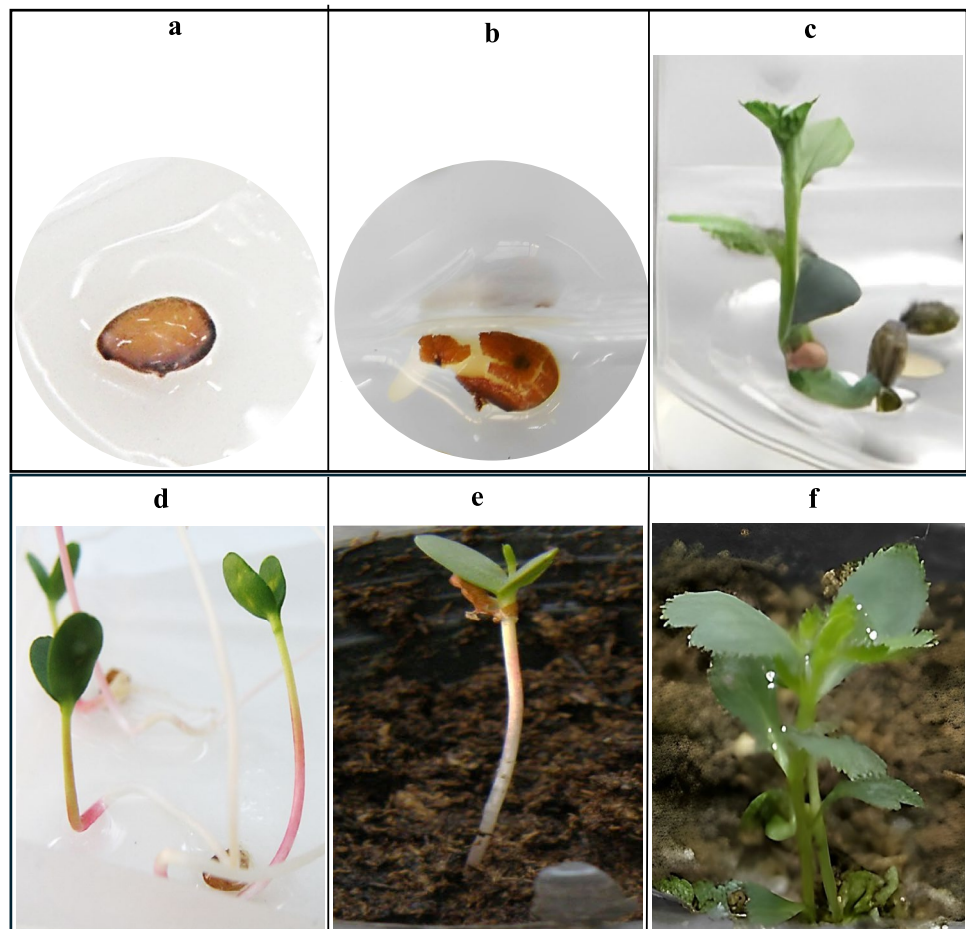
Table 3 Analysis of variance for the effects of media strength (full-MSB5 and $\frac{1}{2}$ MSB5), sinapic acid concentration (0.0, 1, 2, and 3 mg/L), and their interaction on germination percentage of *C. sinica* stratified for 4 weeks at 7 °C

Source of variation	df	MS	F	$P < 0.05$
Sinapic acid concentration	3	1144	20	0.000
Media strength	1	3750	8	0.011
Full-MSB5 x sinapic acid	3	1677	11	0.004
$\frac{1}{2}$ MSB5 x sinapic acid	3	1294	18	0.001
Media strength x sinapic acid	3	1144	20	0.000

Effect of different growth regulators on germination of seeds stratified at 7 °C in ddH₂O substrate

Testing the best growth regulator treatments using water as a growth substrate is a step towards simplifying the germination procedure for future applications. We tested a standard treatment with GA, SA, and GA $\times$ SA $\times$ mTp concentrations on germination. Different growth regulator treatments significantly influenced the germination (Table 4). The mean germination percent ranged from 15 to 46% with different growth regulator treatments (Fig. 6; Table 4 Supplementary

Fig. 4 In vitro germination of *C. sinica* seeds: **a** sterile seeds on MSB5 media; **b** dormancy break and seed germination; **c** six-week-old seedlings cultured on MSB5 supplemented with mTp and BA, each at 0.5 mg/L; **d** eight-week-old in vitro germinated seedlings cultured on sterile ddH₂O supplemented with GA, SA, and mTp (at 200, 1, and 0.5 mg/L, respectively); **e** acclimatization of seedlings after one week; **f** acclimatized seedlings after 2 months



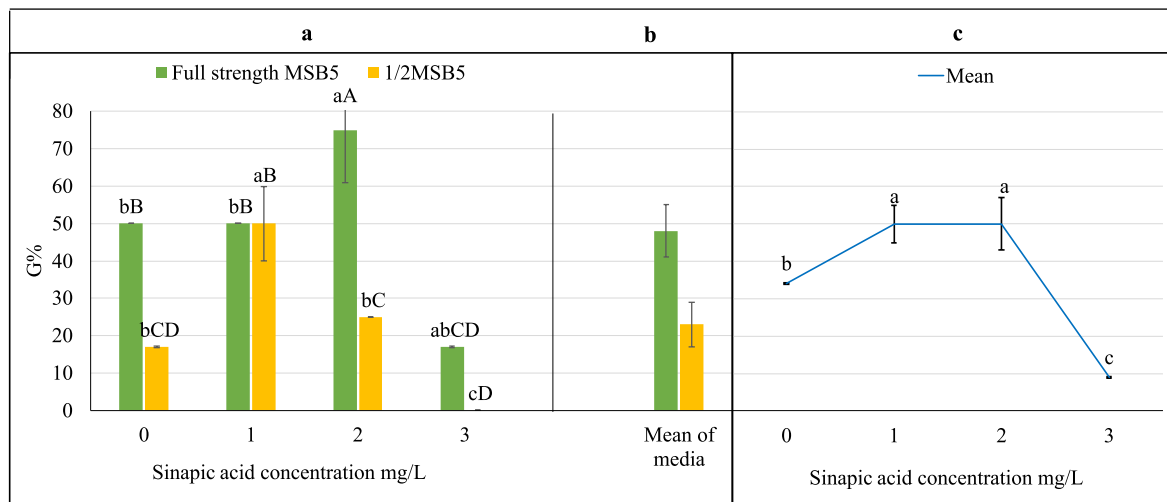


Fig. 5 Germination frequency of *C. sinaica* seeds (mean $\pm$ standard error) as affected by: **a** different sinapic acid (SA) concentrations (0, 1, 2, and 3 mg/L) and media strength (full-strength MSB5, and $\frac{1}{2}$ MSB5); **b** pooled mean of different SA concentrations for each media

type (full-strength MSB5, and $\frac{1}{2}$ MSB5); **c** pooled means of each SA concentration for both media type. Different alphabets indicate significant differences at $P < 0.05$

Table 4 Analysis of variance for the effect of different growth regulators supplemented in H_2O and incubated at $7^\circ C$ on the mean germination percentage of *C. sinaica* seeds cultured under aseptic conditions

Source of Variation	df	MS	F	$P < 0.05$
Treatment	3	578	70	0.000

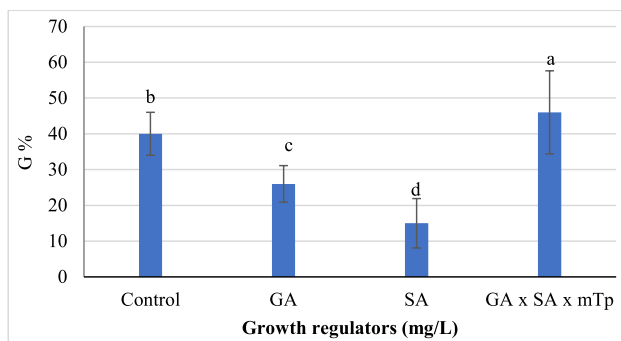


Fig. 6 The effect of different growth regulator treatments (Control, 0.0 growth regulators; GA: 1 mg/L gibberellic acid; SA: 1 mg/L sinapic acid; GA $\times$ SA $\times$ mTp: 200 mg/L GA, 1 mg/L sinapic acid, and 0.5 mg/L meta-Topolin) supplemented in sterile ddH_2O on the frequency of germination of *C. sinaica* seeds. Different alphabets indicate significant differences at $P < 0.05$

data). Stratified seeds cultured on water supplemented with GA (200 mg/L), SA (1 mg/L), and mTp (0.5 mg/L) showed the highest percent (46%) followed by control (40%). Seedlings developed on sterile ddH_2O supplemented with GA, SA, and mTp for two months (Fig. 4d) were acclimatized (Fig. 4e).

Changes in seed physiology during maturity and germination

To understand specific aspects in the physiology of seed maturation and germination, we studied both the primary metabolites and hormonal behavior during the late growing season and after stratification. We also aimed at investigating their potential correlation with germination efficiency. Total soluble sugars, total carbohydrates, and total protein concentrations in seed samples collected from different localities during fruit ripening and seed development and maturation in August, September, and October were determined. Their variation was also assessed. Mature seeds collected during October were cold stratified, and their primary metabolites were determined and compared to their concentrations during their development and maturity in plants.

Variation in primary metabolites

All studied primary metabolites showed significant differences in collection time (month), locality, and their interaction at $P < 0.05$. Only the effect of collection location on total soluble sugars was non-significant (Table 5). Figure 7 and Table 5, supplementary data, shows seed primary metabolites, including soluble sugars (TSS), total carbohydrates (TC), and total soluble proteins (TSP). Soluble sugars decreased in September, then increased in October, and showed the highest content after stratification treatment. For the seeds from L2, increasing the plant's seed age increased TSS. Higher TC contents were estimated in August for the three localities. The locality, L3, showed the highest content (87.70 mg/g DW), followed

Table 5 Analysis of variance for the effect of collection time/date (T) and location (L) and their interaction (L x T) on mean primary metabolites (total soluble sugars, TSS; total carbohydrates, TC; and total soluble proteins, TSP) of *C. sinaica* seeds

Primary metabolites	Source	df	MS	F	$P < 0.05$
TSS	L	2	53.80	1.56	0.230
	T	3	861.63	25.02	0.000
	L x T	6	177.21	5.15	0.002
TC	L	2	382.25	12.19	.000
	T	3	778.84	24.84	0.000
	L x T	6	372.63	11.89	0.000
TSP	L	2	101.73	37.39	0.000
	T	3	595.38	218.83	0.000
	L x T	6	81.12	29.82	0.000

by L2 (65.24 mg/g DW). In September, the content decreased significantly for seeds from L1 and L3, while

a nonsignificant change was observed in L2. In October, while an increase in seed samples from L1 and L3 was detected, the content of carbohydrates in L2 significantly decreased from what it was in the previous month. Stratification pretreatment showed a non-significant increase in seed samples collected from the three locations. The TSP contents increased in September in L1 and L2, then showed a downward trend during October and stratification; for seeds from L3, a downward trend during seed ripening and stratification pretreatment was observed; the lowest TSP contents were detected in stratified seed samples for all three studied localities.

After stratification, only soluble sugars gave their highest concentration (63.96 mg/g DW), while proteins were at a minimal concentration of 8.18 mg/g DW.

During seed maturation, soluble sugars and proteins gave their highest concentrations of 41.4 and 26.25 mg/g DW, respectively, at L1. The highest overall mean of carbohydrates (64.84 mg/g DW) was detected in seed samples from L3.

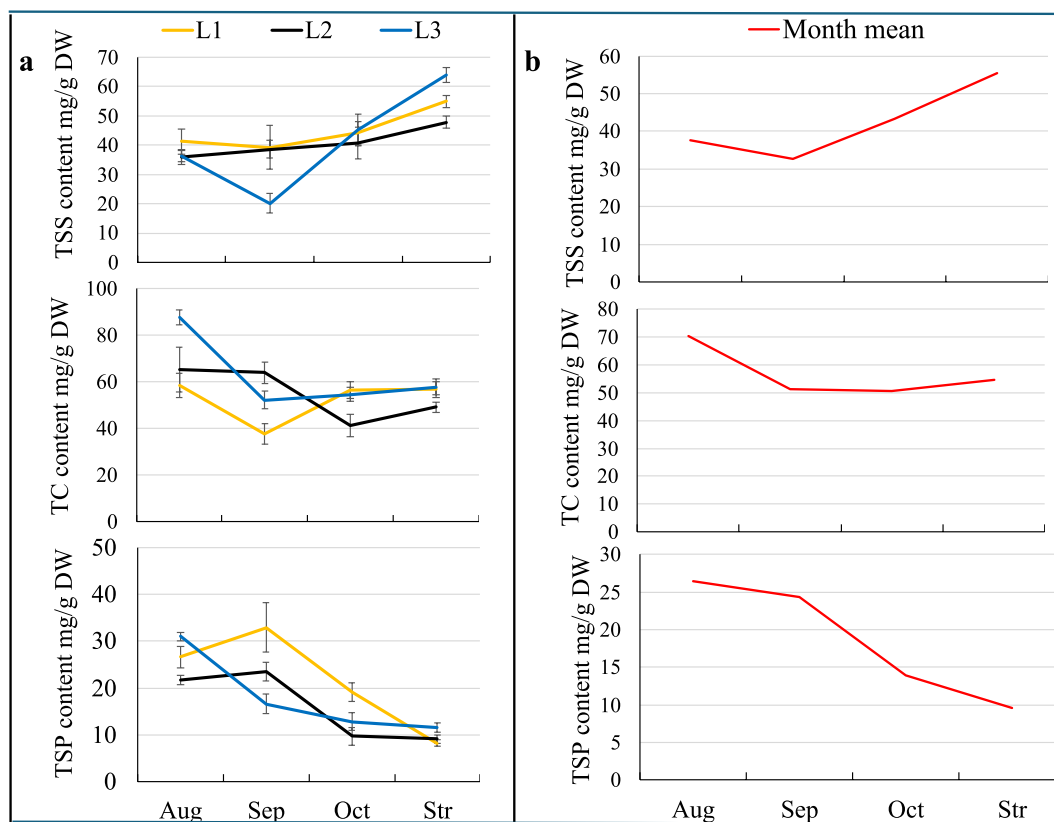


Fig. 7 Variation in the concentration of primary metabolites: total soluble sugars, TSS; total carbohydrates, TC; and total soluble proteins, TSP, expressed as mg/g DW in seeds of *Crataegus sinaica* as affected by collection time (August, Aug; September, Sep; October, Oct; and stratification treatment for October seeds, Str), in the 3 col-

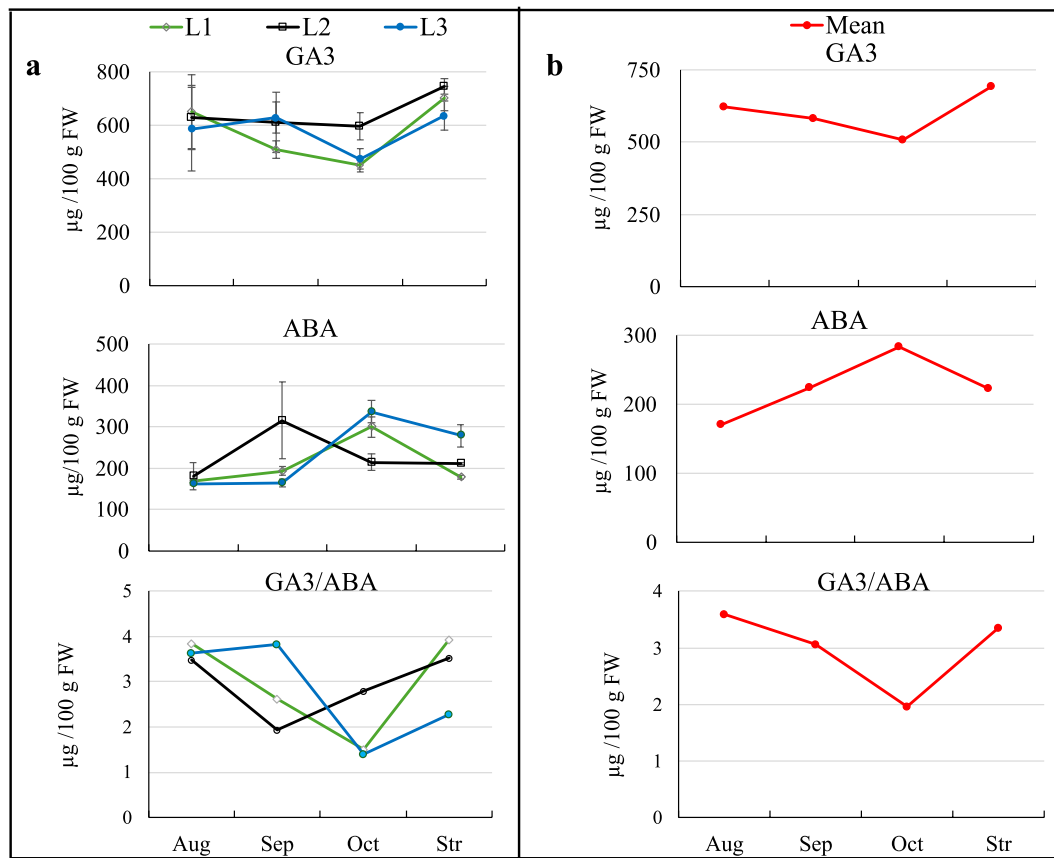
lection sites: Abohamman, L1; Mosa Mountain, L2; and Alragania, L3. **a** Month means in the 3 collection sites **b** Pooled means of different locations over the 3 collection dates and the stratification treatment. Vertical bars represent $\pm$ SE

Table 6 Analysis of variance for the effects of collection time, location, and their interaction on the mean content and ratio of endogenous phytohormones of *C. sinaica* seeds

Phytohormone	Source	df	MS	F	$P < 0.05$
ABA	Collection time	3	19,225.40	4.92	0.008
	Location	2	2144.87	.55	0.585
	Collection time $\times$ Location	6	12,435.39	3.18	0.019
GA ₃	Collection time	3	54,356	2	0.084
	Location	2	17,595	1	0.458
	Collection time $\times$ Location	6	8712	0	0.872
GA ₃ /ABA	Collection time	3	548.18	8.96	0.000
	Location	2	476.11	7.78	0.002
	Collection time $\times$ Location	6	510.39	8.34	0.000

Endogenous phytohormones

A significant effect of both time/date of collection and interaction of collection time with locality on the levels of the endogenous hormones ABA was detected (Table 6). Collection time, locality, and their interaction non-significantly affected the GA content. Figure 8 and Table 6 Supplementary data) presents the changes in total means of endogenous hormone concentration (GA₃, ABA) and their ratios (GA₃/ABA). Time of seed collection enhanced the accumulation of ABA but reduced GA content, irrespective of the locality (Fig. 8a). In October (fully matured seeds), the highest value of ABA was observed, whereas GA recorded its lowest value. Consequently, a negative correlation could be observed between GA₃ and ABA (Fig. 8b). Stratification resulted in minimum values of ABA, while increasing the content of GA₃.

**Fig. 8** Variation in endogenous hormone concentration (GA₃ and ABA μg/100 g FW) and the GA₃/ABA ratio, in the seeds of *C. sinaica* as affected by the collection time (August, Aug; September, Sep; and October, Oct). Stratification treatment was carried out for seeds

collected in October (Str). **a** Means in the 3 collection sites: Abohaman, L1; Mosa Mountain, L2; and Algragania, L3. **b** Pooled means of locations for GA₃, ABA, and GA₃/ABA, over the three collection dates and the stratification treatment

The ratio of endogenous hormones (GA3/ABA) decreased during seed maturation from August to October, while stratification significantly increased the ratios of the same hormones. As shown in Fig. 8b, the endogenous hormone ratios (GA3/ABA) decreased during seed maturation from August to October, whereas stratification increased the GA3/ABA.

Discussion

Crataegus sinaica is an endangered plant species propagated by seeds. No other propagation method has been reported to date. Low germination frequency due to physical and physiological dormancy, as well as extremely slow germination in the wild, presents a significant problem for both the natural spreading and the artificial propagation of such an economically important species. When such factors are coupled to the harsh environment of growth and the impact of climatic change, elaborate research is needed to improve propagation through seeds to maintain naturally occurring variability as found in the wild populations. The present study investigated in vitro seed germination, and some of its possible improvement and controlling factors, as a potential solution to raise germination efficiency. Simple germination procedures for field application have also been addressed.

In the present research, cold stratification at 7 °C enhanced the germination more effectively than at – 20 °C. This may be attributed to appropriate temperature, humidity management, and better water uptake by the seeds (Baskin and Baskin 2004; Yang et al. 2008; Ahmadloo et al. 2017). Cold storage of Sinai hawthorn seeds for 4 weeks improved germination by breaking seed dormancy, stimulating growth regulator content, and increasing seed energy reserves. Storing seeds at 7 °C results in higher energy reserves (TSS and TC), reduced ABA levels by 21.5%, boosted GA production by 37%, and consequently, enhanced germination frequency. Studies on ABA content in *Crataegus* seeds are limited. ABA was shown to inhibit germination in dormant seeds of *C. azarolus* (Qrunfleh 1991), *C. pinnatifida*, and *C. sanguinea* (BingZhen et al. 1998). A decrease in ABA during stratification promotes germination in these species, suggesting that stratification may affect the synthesis or degradation of ABA in the seeds. Furthermore, ABA contents declined sharply during stratification in the deciduous tree species *Davidia involucrata* (Deng et al. 2021). The ABA changes in *Crataegus* seeds during different months and stratification treatments showed a negative correlation with the changes in GA content. The highest ABA content was observed in full-ripened dormant *C. sinica* seeds in October, indicating their importance in seed development and maturation, while the low levels of GA may be associated with seed dormancy or inhibition of germination. Changes

in gene expression, particularly those related to GA and ABA metabolism, influenced the regulation of dormancy in *Arabidopsis* seeds (Footitt et al. 2011). According to Skubacz and Daszkowska-Golec (2017), high GA content in stratified seeds may result from GA biosynthesis in the embryo during storage, promoting radical protrusion during seed germination. Increases in GA3 content in stratified *Crataegus* seeds correlated with increased GA3/ABA ratio. The ABA/GA balance is influenced by genes related to ABA and GA metabolism and signaling (Chen et al. 2020; Ali et al. 2022). Dormancy break and improved rate of germination were associated with increased GA and dynamic hormone ratios and reduced ABA content in *Idesia polycarpa* Maxim (Yanmei et al. 2018), rice (Yang et al. 2019) and in *Tilia* seeds (Wu et al. 2022).

Increased TSS and TC levels in stratified seeds suggest a general enhancement in carbohydrate synthesis and accumulation (Wu and Shen 2021), triggering genes associated with carbohydrate metabolism (Zhang et al. 2022). Stratification increased TSS content by 24.56% for L1, 17.42% for L2, 41.27% for L3, and 28% across all study localities. TSS may play an added critical role to hormonal changes in both seed dormancy and germination. TSS may play a role as an osmoticum that may be combined role with high levels of ABA in dormancy. Mobilization of TSS during germination may provide an energy source, that when coupled to elevated GA3 concentration may have a positive role in dormancy break and commencement of germination. Seed primary metabolites varied greatly with locality and collection time. Fully ripened mature seeds collected in October exhibited an increase in TSS from different localities, suggesting a role in seed longevity and viability during storage (Martínez Maldonado et al. 2015). Similarly, galactinol in Brassicaceae and tomato seeds correlates with longevity (Vidigal et al. 2016).

Applying growth-promoting substances as BA and mTp, or antagonists of ABA as sinapic acid, promoted improved germination. Cytokinins stimulate cell division in the embryo (Kucera et al. 2005) and antagonize the ABA-mediated inhibitory effect (Kucera et al. 2005; Leubner-Metzger and Finch-Savage 2006), specifically by inhibiting *ABI5* transcription (Wang et al. 2011) or promoting *ABI5* protein degradation (Guan et al. 2014), which is involved in ABA signaling during seed maturation and germination. Different types of cytokinins can impact the total cytokinin pool, impacting cytokinin metabolism, signaling, and transport (Bairu et al. 2009; Teixeira da Silva et al. 2020). The application of cytokinin BA or mTp to the culture medium, coupled to seed exposure to stratification at 7 °C, effectively induced the breakage of dormancy in *C. sinaica* seeds. Previous studies showed that cytokinin application promoted in vitro seed germination in *Lotus corniculatus* (Nikolić et al. 2006) and in *Ferula assa-foetida* (Hassani et al. 2009). Sharifi and Pouresmael (2006) also observed

the successful breakage of dormancy in seeds with the use of BA treatment combined with stratification. Meta-topolin treatment was more effective than BA treatment at the same concentration (0.5 mg/L). In this regard, the use of mTp and its derivatives as a cytokinin substitute in plant tissue culture was reported to be effective due to their comparable cytokinin activity (van der Westhuizen 2014; Ahmad and Strnad 2021), the potential for promoting shoot multiplication and growth (Amoo et al. 2011; Stevens and Pijut 2018), lower toxicity, and easier degradation, making them useful for treating physiological problems and morphological abnormalities caused by in vitro culture (Bairu et al. 2009; Amoo et al. 2011).

The concentration of SA and media strength have an interactive effect on seed germination. SA at low concentrations effectively enhanced the germination of stratified seeds, while higher concentrations of SA inhibited it. Considering the media strength, the study suggests that full MSB5 media is more effective for achieving a higher GP in *C. sinaica* seeds compared to half-strength media. The germination percentage was increased by approximately double when using full-strength MSB5 media compared to ½ MSB5. Kim et al. (2019) found that germination in *Pecteilis radiata* was higher in full-strength MS media. In contrast, *Sedirea japonica* seedling growth was higher in half-strength MS media than in full-strength media (An et al. 2021). This suggests that nutrient requirements vary among plant species, and the concentration of nutrients in the media significantly influences germination and growth in these species. Sinapic acid stimulates seed germination by inhibiting ABA synthesis (Deng et al. 2021). According to Bi et al. (2017), SA is the primary phenolic compound channeled into seeds and converted into SA esters, regulating ABA glycosylation and plant ABA homeostasis. It enhances the accumulation of sinapoyl choline, a crucial compound for successful seed germination.

Combining GA, SA, and mTp in a water growth substrate had a positive impact on GP. Shu et al. (2016) and Bi et al. (2017) found that gibberellic acid and sinapic acid positively stimulated the germination process by counteracting ABA-mediated inhibition of seed germination (Shu et al. 2016; Bi et al. 2017). A previous study on *C. sinaica* found GA treatment for hawthorn seeds yielded the highest germination rate (Sümbül et al. 2024). Moreover, GA promotes embryonic cell elongation (Hauvermale and Steber 2020), inhibits the expression of ABA-responsive genes (Skubacz and Daszkowska-Golec 2017), and stimulates enzymes for energy mobilization and degradation of endosperm cell walls, thereby facilitating radicle protrusion (Skubacz and Daszkowska-Golec 2017; Hauvermale and Steber 2020).

In the present research, coupling the growth regulator regime identified using in vitro culture with long term cold

imbibition of seeds using sterile distilled water, significantly enhanced dormancy break and seed germination. This modification could offer an inexpensive practical and simple protocol for seed germination of *C. sinaica*, that may be extended to seeds of other recalcitrant species. The importance of such an approach is in simplifying practical procedures and cost feasibility for application in plant propagation and growth. It also suggests that *C. sinaica* seeds contain enough reserves that only need to be mobilized for enhancing dormancy break and germination.

Conclusion

The study presents the first report of the successful germination in the recalcitrant, economically important Sinai hawthorn. Cold stratification, cytokinin type and concentration, and sinapic acid, all support efficient germination. Mobilization of seed reserves and the balance between GA and ABA levels were key variables for the regulation of seed dormancy and promoting seed germination. The use of water as a growth substrate instead of nutrient media is significant for future greenhouse trials to simplify the process of germinating and propagating species, at low cost, with simple technical capabilities. The successful development of a simple efficient applicable technique for seed germination could greatly aid in the conservation efforts and sustainable cultivation and restoration of this valuable tree species. Further research is needed to explore additional factors that may influence dormancy break and germination success, as well as, developing strategies for enhanced seedling growth under both controlled and field conditions.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11240-025-03006-5>.

Author contributions AE designed the research work. HA and SM conducted the sample preparation, material analysis, and data collection. HA performed the data analysis and wrote the manuscript with guidance, correction, proofreading, and valuable edits by AE. All authors read and approved the final manuscript.

Funding Open access funding provided by The Science, Technology & Innovation Funding Authority (STDF) in cooperation with The Egyptian Knowledge Bank (EKB). No funding, grants, or other support was received for conducting this research.

Data availability All data generated and analyzed during this study are included in this article and are available from the corresponding author upon reasonable request.

Declarations

Competing interests The authors declare that there is no conflict of interest.

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