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Research paper

High efficiency stratification of apple cultivar Ligol seed dormancy by phytohormones, heat shock and pulsed radio frequency

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ABSTRACT

The aim of the study was to improve the effect of stratification of apple "Ligol" seeds by application of selected compounds, phytohormones, and physical methods For this purpose the seeds were stratified at 3 °C in distilled water or in the presence of potassium nitrate (KNO_3), ethephon (ET), carbon monoxide (CO), hydrogen peroxide (H_2O_2), a mixture of KNO_3, ET, CO, H_2O_2, gibberellins (GA_3), 6-benzylaminopurine (BAP), jasmonic acid (JA), salicylic acid (SA) and a mixture of SA, GA_3, BAP, JA, nitric oxide (NO), hydrogen chloride (HCL). Arranged protocols included various durations and combinations of selected compounds and phytohormones as well as laser and red light, heat shock − 2 h heat shock (45 °C) and Pulsed Radio Frequency (PRF) were investigated by germination tests and the activity of selected enzymes, gas exchange and index of chlorophyll in leaves.

The obtained results showed the possibility to shorten more effectively the time of the apple 'Ligol' dormancy removal by treatments of the stratified seeds at 3 °C with different biological and physical methods Selected compounds and phytohormones acted collectively as a regulatory complex controlling the course of release from dormancy. Physical methods (PRF and heat shock) additionally contributed to dormancy breakage. Duration of phytohormones or compounds impacts during stratification should be prolonged to minimum 7 days to assure more balanced conditions of the regulatory complex for the acceleration of dormancy a removal. The most beneficial results were obtained after seed stratification for 7 days on filter paper moistened in KNO_3 + Etephon + CO + H_2O_2 at 3 °C, and then on filter paper moistened in phytohormones (GA_3 + BAP + JA) till the end of seed germination (3 °C). The application of this protocol could be a very useful tool in a shortening the apple breeding cycle since the period of removing dormancy was reduced by 38 days in comparison to stratified in water. PRF has also the additive role in breaking dormancy of apple 'Ligol' seed. Positive effects of compounds and phytohormones applied during stratification remarkably accelerated the growth of developed from them seedlings. Further research is needed to optimize stratification methods with appropriate contents and concentrations of compounds and phytohormones combined with PRF exposure.

1. Introduction

The seed germination ability can be regulated by environmental signals, including endogenous and combination with synergistic and antagonistic effects (Arc et al., 2013). Seed dormancy is determined by genetic and environmental factors (Graeber et al., 2012). Apple seeds after harvest are in a state of deep dormancy and are able to germinate after a period of after-ripening process under the conditions of so-called cold stratification (Żarska-Maciejewska, 1976). Some of the plant hormones, like gibberellins (GA_3), cytokinins like 6-benzylaminopurine (BAP), jasmonic (JA), ethylene (ET), Salicylic acid (SA) are taking part in removing apple seed dormancy (Bogatek et al., 2002; Chitnis et al., 2014; Finch-Savage and Leubner-Metzger, 2006; Lewak, 2011; Ranjan and Lewak, 1994). Gibberellins are known as growth-promoting hormones, being involved in several processes during plant development, such as shoot growth, flower development, dormancy release and seed germination (Hilhorst and Karssen, 1992; et al., 2012Linkies and Leubner-Metzger, 2012; Sińska, 1989). Jasmonic acid also stimulated

Abbreviations: KNO_3, potassium nitrate; ET, ethephon; CO, carbon monoxide; H_2O_2, hydrogen peroxide; GA_3, gibberellin A3; BAP, 6-benzylaminopurine; CKs, Cytokinins; JA, jasmonic acid; SA, salicylic acid; NO, nitric oxide; HCL, hydrogen chloride; PRF, Pulsed Radio Frequency

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germination of dormant apple seeds (Yildiz et al., 2007). Similarly, the role of cytokinins in the removal of embryonic dormancy in apple seeds has been documented by changes in the activity of endogenous hormones and also by their effects on germination of isolated embryos (Zhang and Lespinasse, 1991). Salicylic acid (SA) can be associated with the regulation of seed dormancy and germination by modulating the cellular level of reactive oxygen species (ROS), which act as signaling molecules in the alleviation of seed dormancy in both monocot and dicot species (Chitnis et al., 2014). However, the effect of exogenous SA on seed germination is influenced largely by its concentration. Arc et al. (2013) have found that ethylene, a gaseous plant hormone can prevent the inhibitory effects of ABA on endosperm cap weakening, thereby facilitate endosperm rupture and radicle emergence of *Brassicaceae* seeds. The application of KNO_3 is often a primarily compound used in seed-testing laboratories for many years without a good explanation for its action mechanism. It is well documented that increases the germination of photo-dormant seeds (Shanmugavalli et al., 2007). In some species, seed dormancy can be regulated by red or laser light (Leinonen and Chantal de, 1998). The mentioned so far research has indicated that it is possible to reduce the dormancy time release of an apple seed, but the effectiveness of the presented methods is still unsatisfactory since too the long period of germination led to an extended time of the new breeding varieties.

For the first time, in the field of seed science and in order to break apple seed dormancy, application of Pulsed Radio Frequency (PRF) is proposed, which emits electric fields generated by an RFG 3C PLUS lesion generator. PRF, for over last two decades has been applied in medicine in chronic pain therapy (Cosman, 2005). Since this technique is employed to treat pain, movement, and mood disorders, presumably would exert a beneficial influence on crucial blocked processes involved in breaking seed dormancy.

The aim of the present study was to accelerate apple 'Ligol' seeds release from dormancy and juvenile plant growth by application of selected compounds, phytohormones as well as physical methods. For this purpose stratification at 3 °C was enriched with various combinations (protocols) of KNO_3, ET, CO, H_2O_2, GA_3, BAP, JA, SA, NO, HCL, laser and red light, heat shock and PRF.

2. Materials and methods

2.1. Plant material

The research was conducted on apple (*Malus domestica*) seeds and seedlings belonging to a late-maturing variety of 'Ligol'. The seeds were received in the autumn of 2013–2016 from the Experimental Orchard of the Research Institute of Horticulture in Skierniewice. Apple seeds were extracted from fruits directly after harvest and were dormant and not able to germinate within 7 days at 3–30 °C.

2.2. Breaking of seed dormancy

The collected seeds were subjected to stratification at a constant temperature of 3 °C for 90 days in darkness. The control seeds (with coats) were placed in 6 cm diameter Petri dishes (50 seeds per dish in three replicates), on two layers of filter paper moistened with distilled water. The other batches of seeds, instead of water, were subjected to biological methods (selected compounds, phytohormones) and physical treatments (Pulsed Radio Frequency, short-termed heat shock, red and laser light) and then transferred to a chamber manufactured by Binder GmbH, German, prior to sowing in greenhouse, in order to obtain seedlings. The research was divided into six independent experiments.

1 The seeds were subjected to stratification on the filter paper moistened with potassium nitrate (KNO_3), ethephon (ET), hydrogen peroxide (H_2O_2) or aerated with carbon monoxide (CO) and a mixture of KNO_3, ET, CO, H_2O_2. KNO_3 was used in the concentration of 0,2%. ET was applied as a seed treatment with Agrostym 480 SL (0.2%). CO was obtained by application of hematin, which is a donor of CO in a concentration of 2 mM for 3 h in the light (Gniazdowska et al., 2010). H_2O_2 was applied in the concentration of 3% for 24 h

2 Seed stratification was conducted on the filter papers moistened with the following phytohormones: salicylic acid (SA), gibberellin A3 (GA_3), 6-benzylaminopurine (BAP), jasmonic acid (JA) and a mixture of SA, GA_3, BAP, JA. SA was applied in the concentration of 500 mM, gibberellin A3 (GA_3) in 5 mM, 6-benzylaminopurine (BAP) in 25 mg/l, the jasmonic acid (JA) in 10^{-3}M. All phytohormones used as a mixture of SA, GA_3, BAP and JA were applied simultaneously. The optimal concentrations of the selected growth regulators were chosen on the basis of previously conducted experiments.

3 The apple seeds were subjected to scarification in HCL and stratification at 3 °C in the presence of nitric oxide (NO), laser light and red light. Scarification of seed coats was conducted for 5 min with hydrogen chloride (HCL) in the concentration of 1% and then transferred to stratification at 3 °C. NO was obtained by application of sodium nitroprusside (SNP) in the concentration of 5 mM for 3 h (Gniazdowska et al., 2007). Red light was applied to stratified seeds for 7 days. He-Ne laser light was applied using parameters of a wavelength of 632.8 nm and a surface power density of 3 mW cm-2–for 5 min.

4 The seeds were subjected to stratification both in the presence of the mixture of compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (GA_3 + BAP + JA) in concentrations mentioned above. The experiment was carried out in the following protocol: Control − seeds stratified at 3 °C on the filter papers moistened with distilled water. I. − 1 day (d) seed soaking in the compound mixture (20 °C) and then germination on the filter paper moist. with phytohormones. II. − 7 d seed stratification on filter paper moist. with phytohormones mixture and then germination on filter paper moist. with compounds. III. − 7 d seed stratification on filter paper moist. with compounds and then with phytohormones. IV. − seed germination on the filter paper moist. simultaneously with all selected compounds and phytohormones.

5 Seeds were subjected to stratification in phytohormones and compounds and in the meantime exposed to the short-termed heat shock. The experiment was carried out in the following protocol: Control − seeds stratified at 3 °C on the filter papers moistened with distilled water. I. − 7 d seed stratification on filter paper moist. with H_2O, then the exposure to heat shock for 2 h (45 °C), afterwards germination on the filter paper moist. with H_2O. II. − 1 d seed soaking in compounds and then stratification for 7 d on filter paper moist. with phytohormones, then the exposure to heat shock (45 °C) for 2 h and returned to stratification on the filter paper moist. with phytohormones. III. − 1 d seed soaking in compounds and then stratification for 7 d on the filter paper moist. with phytohormones, then the exposure to 20 °C for 7 d and returned to stratification with phytohormones. IV. − 1 d seed soaking in compounds and then incubation for 7 d on the filter paper moist. with phytohormones at 20 °C and returned to stratification on the filter paper moist. with phytohormones.

6 Stratified apple seeds were treated with Pulsed Radio Frequency (PRF) and then returned to stratification in the presence of selected phytohormones and compounds. The experiment was conducted in the following protocol: Control − seeds stratified at 3 °C on the filter papers moistened with distilled water. I. − 1 d seed soaking in H_2O, then the exposure for 1 h to PRF 25 V/4 Hz/20 ms and stratification on the filter paper moist. with H_2O. II. − 1 d seed soaking in H_2O, then the stratification on the filter paper moist. with H_2O. III − 1 d seed soaking in compounds, then 7 days stratification in phytohormones, then the exposure for 1 h to PRF 25 V/4 Hz/20 ms and returned to stratification on the filter paper moist. with H_2O. IV. 1 d

seed soaking in compounds, then 7 days stratification in phytohormones and returned to stratification on the filter paper moist with H_2O.

PRF was applied using an RFG 3C PLUS lesion generator (Radionics, Burlington, MA, U.S.A). For this purpose, the moistened seeds were placed in a Plexiglas container, immersed in water and squeezed with a metal grid connected to a metal spindle. Electrode was connected to the spindle to allow free flow

of PRF. On the basis of preliminary experiments, the seeds were subjected to the PRF exposure for 1 h, at a constant voltage of 25 V, pulse repetition rate 4 Hz (pulses per second) with pulse duration 20 ms. The applied PRF settings were incorporated into protocol I and III of 2.2.5 experiment.

2.3. Evaluation of the seed treatment impact on germination and seedling growth

2.3.1. Dynamics of seed germination

Stratified apple seeds on filter paper moistened with distilled water, compounds and phytohormones were evaluated in respect of the number and dynamics of germinated seeds. A seed was regarded as germinated when the radicle emerged from the pericarp. Germinated seed was counted every day to estimate the dynamics of seed germination and the percentage of seed germination.

2.3.2. Dynamics of seedlings growth

After stratification the germinating seeds were sown individually in the pots containing the medium consisted of peat and sand mixed in a proportion 1:1 (v/v). The pots were transferred to greenhouse conditions. Plant height was measured from the soil surface to the top of the plant, at 2–3 week intervals, with a scaled meter, which was 50 cm long. At each measurement, 10 individuals in the middle rows were measured in three replicates.

2.3.3. Gas exchange in leaves

Net photosynthetic rate (PN), transpiration (E), stomatal conductance (Gs), and intercellular CO_2 concentration (Ci) were measured using the portable photosynthesis measurements system TPS-2 (PP Systems, USA) (Kalaji et al., 2014). Measurements were provided under greenhouse conditions at the end of July on fully developed leaves at morning hours (08:00–11:00 h). The temperature was about 20–25 °C, air humidity about 70–80%, and light intensity ca. 1100–1300 μmol (photon) $m^{-2}\,s^{-1}$.

2.3.4. Index chlorophyll content

Index of chlorophyll content (CCI) in leaves was evaluated non-destructively using portable Minolta SPAD-502 chlorophyll meter (Konica Minolta, Japan) and expressed in SPAD units (Grzesik and Romanowska-Duda, 2014). Five readings were obtained from individual leaves. During measurements with the SP502 CE, the sensor head was shaded with the operator's own body as recommended by the manufacturer to avoid direct sunlight from reaching the instrument.

2.3.5. Activities of acid (pH = 6) and alkaline (pH = 7.5) phosphatase and RNase

The physiological activity of plants was studied by measuring the activity of acid (pH = 6.0) and alkaline (pH = 7.5) phosphatase and RNase. Acid (pH 6) (EC 3.1.3.2) and alkaline (pH 7.5) (EC 3.1.3.1) phosphatase [mU g^{-1}(FM) min^{-1}] and RNase (EC 3.1.27.5) [mU g^{-1} (FM) min^{-1}] in the leaves were examined according to the methods described by Knypl and Kabzińska (1977).

2.3.6. Dehydrogenases activity

Dehydrogenases activity were determined as the frequent, reliable and good marker for scoring seed vigor classes. The method used for measuring the activity of dehydrogenases is based on the fact that 2,3,5-triphenyl tetrazolium chloride (TTC) interacts with the reduction processes of living cells and accepts hydrogen from dehydrogenases. By hydration of the TTC a red, stable and non-diffusible substance, triphenyl formazan is produced in living cells (Pandey, 1989). 0.2 g of imbibed for 24 h in H_2O_2 seeds were placed in Eppendorf tubes, ground and incubated in 1 ml of 0.1 M sodium phosphate buffer, pH 7.2 containing 0.7% (w/v) of 2,3,5-triphenyl tetrazolium chloride at 25 °C for 24 h. After that time samples were centrifuged (5 min; 5000 x g) and the pellet was extracted six times with 1 ml of acetone. The solution absorbance was measured at 488 nm. A standard curve was prepared from a known concentration of formazan. Each determination was made four times.

2.3.7. Statistical analyses

The conducted experiments with the determination number and dynamics of germinated seeds, seedlings growth were repeated three times. Gas exchange and index of chlorophyll content, the activity of acid (pH 6.0) and alkaline (pH 7.5) phosphatase, RNase and dehydrogenases activity were repeated four times. The means of chosen parameters were grouped employing Duncan's test at the α = 0.05 significance level.

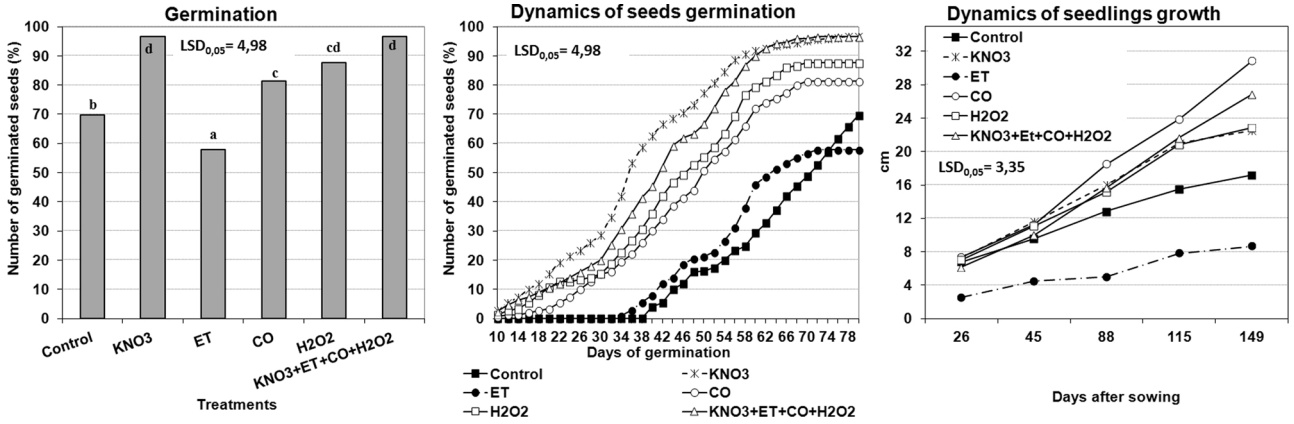

Fig. 1. The effect of potassium nitrate (KNO_3), ethephon (ET), carbon monoxide (CO), hydrogen peroxide (H_2O_2), a mixture of KNO_3, ET, CO, H_2O_2 applied during seed stratification at 3 °C on percentage and dynamics of germination and growth of apple 'Ligol' plants.

Table 1
Gas exchange and index of chlorophyll content in 'Ligol' apple leaves, as affected by potassium nitrate (KNO_3), ethephon (ET), carbon monoxide (CO), hydrogen peroxide (H_2O_2), and a mixture of KNO_3, ET, CO, H_2O_2 applied during seed stratification at 3 °C.

Treatments	Net photosynthesis (μm CO_2 m^{-2} s^{-1})	Transpiration (nmol H_2O m^{-2} s^{-1})	Stomatal conductance (nmol H_2O^{-1} M^{-2} s^{-1})	Intercellular concentrate CO_2 (μmol CO_2 air mol^{-1})	Index of chlorophyll content in leaves
Control	1.70b	1.30b	159a	367c	27.9a
KNO_3	3.80e	2.79e	891d	315a	46.0d
ET	1.50a	1.22a	145a	388c	25.9a
CO	2.77c	1.38c	743b	343b	37.3b
H_2O_2	2.80c	1.65d	827c	344b	42.1c
KNO_3 + ET + CO + H_2O_2	3.30d	1.71d	889d	319a	45.1 cd
$LSD_{0.05}$	0.11	0.06	35	22	3.3

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

3. Results

3.1. The effect of KNO_3, ET, CO, H_2O_2, a mixture of KNO_3, ET, CO, H_2O_2 in combination with seed stratification (3 °C) on apple seed germination, plant growth, and physiological activity

Seeds collected directly after harvest from fruits in November and stratified in water (served as a control) germinated relatively poor, for a long period and with low uniformity. The onset of germination occurred at the 38th day and its end at the 78th day of seed imbibition and only 70% of them germinated (Fig. 1). The seedlings developed from control seeds grew relatively slowly and the final plant height (after 149 days from sowing) was 17 cm. Application of selected compounds, except ET, in combination with stratification, increased percentage and dynamics of seeds germination as well as the height of developed seedlings. The most pronounced effects were obtained after treatments with KNO_3 and with a mixture of KNO_3, ET, CO and H_2O_2. Due to stratification (3 °C) in the presence of KNO_3 (0,2%) dormancy of apple seeds was substantially reduced, which was expressed by the increased percentage of germinated seeds from 70 to 90%. The onset of germination of such treated seeds was accelerated by 28 days in comparison to control. The end of seed germination occurred 8 days earlier than control.

Application of KNO_3 and a mixture of KNO_3, ET, CO, H_2O_2 during stratification significantly increased physiological activity of seedling's cells and tissues, which was expressed by the greater activity of net photosynthesis, transpiration, and stomatal conductance, index of chlorophyll content in the leaves, and lower intercellular CO_2 concentration (Table 1).

3.2. The effect of SA, GA_3, BAP, JA and a mixture of SA, GA_3, BAP, JA with combination with stratification (3 °C) on apple seed germination, plant growth, and physiological activity

Application of the majority of the examined plant hormones resulted in increased the efficiency of stratification (3 °C) (Fig. 2). The most pronounced effects were obtained after GA_3 treatment. Due to such treatment germination of seeds increased up to 93% within 54 days of imbibition. Similar results were obtained after stratification in the presence of a mixture of SA, GA_3, BAP, and JA. However, application of SA decreased the percentage of germinated seeds although dynamics of germination was significantly enhanced in comparison with control. Plant height also significantly increased in response to GA_3, BAP, JA and a mixture of SA, GA_3, BAP and JA. The final height (after 149 days from sowing) of seedlings developed from seeds stratified in GA_3 and in a mixture of SA, GA_3, BAP, JA increased by7 and 3 cm over control plants, respectively.

Application of GA_3, BAP, JA and a mixture of SA, GA_3, BAP, JA during stratification beneficially affected the activity of net photosynthesis, transpiration, and stomatal conductance and index of chlorophyll content in the leaves. Stomatal conductance was especially stimulated by GA_3. In comparison to control GA_3 treatment increased this parameter by 500%. On the contrary, the intercellular CO_2 concentration was lowered by these phytohormones applications (Table 2).

3.3. The effect of NO, laser light and red light applied during seed stratification (3 °C) and scarification of seed coats for 5 min with HCL on apple seed germination, plant growth, and physiological activity

Treatments with NO and laser or red light increased germination of stratified seeds. The application of NO, by using SNP (a donor of NO), resulted in the most substantial increased percentage, dynamics of germination as well as in dynamics of seedlings growth in comparison

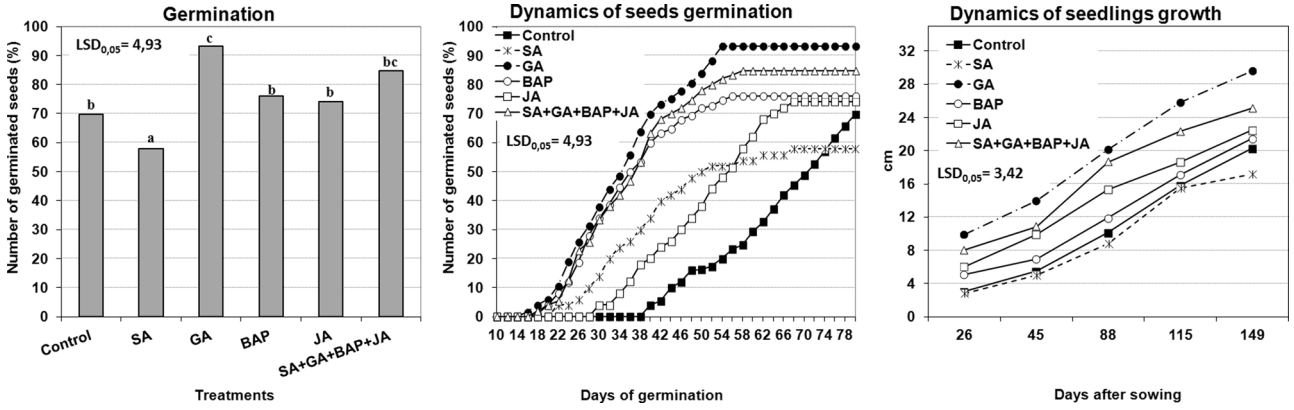

Fig. 2. The effect of salicylic acid (SA), gibberellin A3 (GA_3), 6-benzylaminopurine (BAP), jasmonic acid (JA) and a mixture of SA, GA_3, BAP, JA applied during seed stratification at 3 °C on percentage, dynamics of germination and growth of apple 'Ligol' plants.

Table 2
Gas exchange and index of chlorophyll content in 'Ligol' apple plants, as affected by salicylic acid (SA), gibberellin A3 (GA_3), 6-benzylaminopurine (BAP), jasmonic acid (JA) and a mixture of SA, GA_3, BAP, JA applied during seed stratification at 3 °C.

Treatments	Net photosynthesis (μm CO_2 m^{-2} s^{-1}	Transpiration (nmol H_2O m^{-2} s^{-1})	Stomatal conductance (nmol H_2O^{-1} M^{-2} s^{-1})	Intercellular concentrate CO_2 (μmol CO_2 air mol^{-1})	Index of chlorophyll content in leaves
Control	1.70b	1.30b	159a	356d	27.9a
SA	1.59a	1.22a	141a	367d	25.0a
GA_3	2.90d	1.75e	987d	246a	53.9d
BAP	2,16b	2,45c	640b	330c	37.2b
JA	2,09b	2,41c	687b	332c	35.9b
KNO_3 + ET + CO + H_2O_2	2.70c	1.61d	793c	291b	40.1c
LSD $_{0.05}$	0.10	0.06	34	24	3.6

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

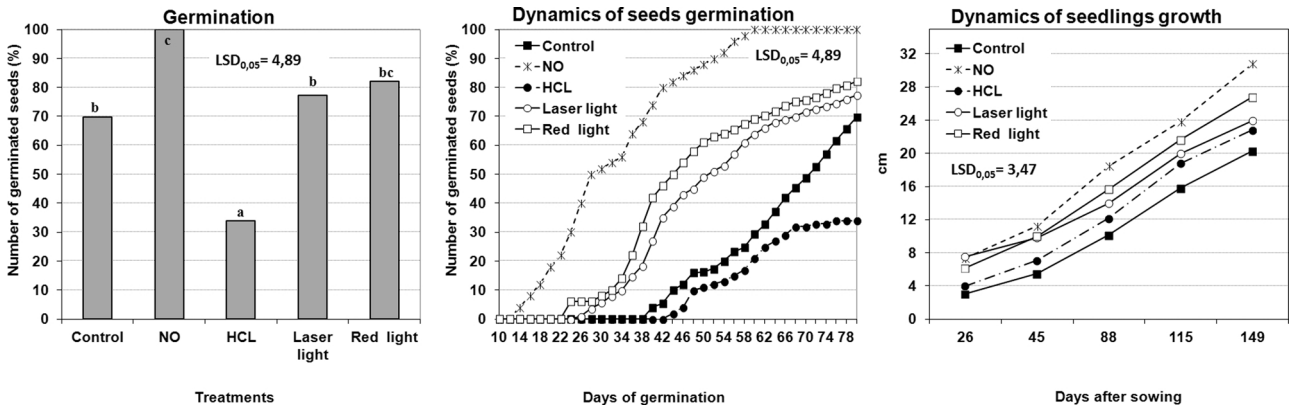

Fig. 3. The effect of nitric oxide (NO), laser light and red light in combination with stratification(3 °C) and scarification of seed coats for 5 min with hydrogen chloride (HCL) on percentage, dynamics of germination and growth of apple 'Ligol' plants. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with control and treatments with laser light, red light and hydrogen chloride (HCL) (Fig. 3). Upon scarification and NO application, seed germination began after 12 days from the beginning of imbibition. Seeds completed germination in 100% within 62 days of imbibition. Similarly, the growth of seedlings developed from seeds stratified (at 3 °C) in combination with NO fumigation was significantly enhanced in comparison with control. Laser and red light improved also dynamics of seed germination and number of germinated seeds, however in case of the percentage of seed germination the improvements were not significant. Application of HCL explicitly decreased both examined germination parameters and dynamics of seedlings growth implying that it may damage seed coats or surrounding tissue of embryos.

Gas exchange and index of chlorophyll content in leaves showed the same dependencies as in the case of percentage, dynamics of seed germination and seedlings growth (Table 3). Especially, a great increase was observed in the stomatal conductance under the influence of NO. Similarly, NO increased index of chlorophyll content in leaves (by 53%) in comparison to control.

3.4. The effect of the mixture of the selected compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (GA_3 + BAP + JA) with a combination of stratification on apple seed germination, plant growth, and physiological activity

Apple seeds subjected to the mixture of selected compounds and phytohormones in various combinations promoted percentage of seed germination, dynamics of seedling growth and accelerated the growth of seedlings, exhibited by higher plants in comparison to control (Fig. 4). The most beneficial effect was obtained after application of selected compounds and phytohormones according to protocol III i.e. 7 days on filter paper moistened in compounds (KNO_3 + Etephon + CO + H_2O_2) at 3 °C, and then on filter paper moistened in phytohormones (GA_3 + BAP + JA) till the end of seed germination (3 °C). Due to such treatments, the germination percentage was increased up to

Table 3
Gas exchange and index of chlorophyll content in 'Ligol' apple plants as affected by nitric oxide (NO), laser light and red light applied during seed stratification at 3 °C and scarification of seed coats for 5 min with hydrogen chloride (HCL).

Treatments	Net photosynthesis (μm CO_2 m^{-2} s^{-1}	Transpiration (nmol H_2O m^{-2} s^{-1})	Stomatal conductance (nmol H_2O^{-1} M^{-2} s^{-1})	Intercellular concentrate CO_2 (μmol CO_2 air mol^{-1})	Index of chlorophyll content in leaves
Control	1.70b	1.30b	159a	356c	26.9b
NO	2.20d	1.44d	230c	193a	41.3 d
HCL	1.60a	1.23a	155a	397d	22.7a
Laser light	2.50e	1.71e	161ab	320b	27.9bc
Red light	2.00c	1.37c	194b	308b	29.9c
LSD 0.05	0.09	0.06	34	24	2.3

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

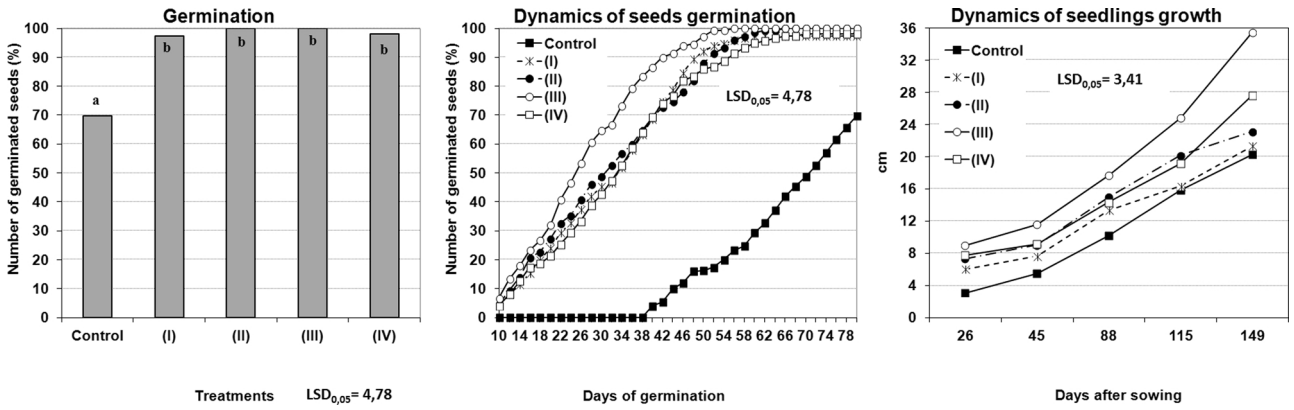

Fig. 4. The effect of selected phytohormones and compounds applied during seed stratification at 3 °C on percentage, dynamics of germination and growth of apple 'Ligol' plants. For the treatments, the mixture of compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (GA_3 + BAP + JA) was selected. The experiment was carried out in the following protocol:.
Control – seeds stratified at 3 °C on the filter papers moistened with distilled water.
I. – 1 day (d) seed soaking in compound mixture (20 °C) → stratification in phytohormones.
II. – 7 d seed stratification on filter paper moist. with phytohormones mixture → stratification in compounds.
III. – 7 d seed stratification on filter paper moist. with compounds → stratification in phytohormones.
IV. – seed stratification on the filter paper moist. simultaneously with all selected compounds and phytohormones. For explanation (I, II, III, IV) please refer to Materials and methods.

Table 4
The activity of acid (pH 6.0) and alkaline (pH 7.5) phosphatase, RNase and dehydrogenases as affected by treatments with mixtures of selected phytohormones and compounds applied during seed stratification at 3 °C. Seeds treatments were carried out according to protocol shown in Fig. 4.

Treatments	Phosphatase (pH 6.0)	Phosphatase (pH 7.5)	RNase	Total dehydrogenases [mg (formazan) g^{-1}(seed FM)]
	[U g^{-1}(FM)]	[U g^{-1}(FM)]	[U g^{-1} (FM)]	
Control	0.28a	0.10a	1.50a	0,9 a
I	0.36b	0.13b	2.01b	1,9 b
II	0.39b	0.14b	2.25bc	2,2 c
III	0.46c	0.18c	2.39c	2,6 d
IV	0.38b	0.14b	2.25bc	2,4 c
LSD 0.05	0.03	0.02	0.30	0,2

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

100% and the period of seed germination reduced to 50 days. Positive effects of compounds and phytohormones applied during stratification were visible not only during germination but also remained notable within 5 months of seedlings growth in pots (Fig. 4). Due to GA_3 inclusion during seed stratification the height of developed from them seedlings increased by 16 cm in comparison to control.

These events were preceded by the increased activity of acid (pH = 6.0) and alkaline phosphatase (pH = 7.5), RNase, total dehydrogenase and followed by the activity of net photosynthesis, transpiration, stomatal conductance, index of chlorophyll content in leaves, as well as decreased intercellular CO_2 concentration (Tables 4 and 5).

3.5. The effect of the heat shock and a mixture of the selected compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (GA_3 + BAP + JA) with a combination of stratification on apple seed germination, plant growth, and physiological activity

Short-term heat shock (at 45 °C for 2 h) applied alone during stratification (protocol I) and together with selected compounds and phytohormones (protocol II) significantly stimulated percentage and dynamics of seed germination as well as seedling growth (Fig. 5). However, application of the selected compounds and phytohormones (protocol III and IV) appeared to exert more beneficially on examined parameters than used together with heat shock. Apple seeds treatments according to protocol III were the most effective in respect of dormancy release. According to this procedure, the seeds kept at 20 °C were soaked for 1 day in KNO_3 + Etephon + CO + H_2O_2, then at 3 °C for 7 days on filter paper moistened in GA_3 + BAP + JA. Afterward, the seeds moistened with the same phytohormones were transferred to 20 °C for the next 7 days and finally until the end of germination were returned to 3 °C. In response to treatments according to protocol III, the seeds germinated in 100% within 58 days. The positive effects maintained after seedlings emergence since within 149 days from seed sowing, the seedlings height was increased by 13 cm in comparison to control.

Due to application of heat shock, selected compounds and

Table 5
Gas exchange and index of chlorophyll content in 'Ligol' apple plants. as affected by compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (SA + GA_3 + BAP + JA) applied during seed stratification at 3 °C. Seeds treatments were carried out according to protocol shown in Fig. 4.

Treatments	Net photosynthesis (μm CO_2 m^{-2} s^{-1}	Transpiration (nmol H_2O m^{-2} s^{-1})	Stomatal conductance (nmol H_2O^{-1} M^{-2} s^{-1})	Intercellular concentrate CO_2 (μmol CO_2 air mol^{-1})	Index of chlorophyll content in leaves
Control	1.70a	1.30a	159 a	356b	27.9a
I	1,81b	1,44c	194b	357b	31,9b
II	1,86b	1,36b	165ab	350b	39,9c
III	2,5d	1,71e	886c	323a	41,3d
IV	2,07c	1,60d	161bab	338ab	31,7b
LSD 0.05	0,09	0,06	35	24	3,3

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

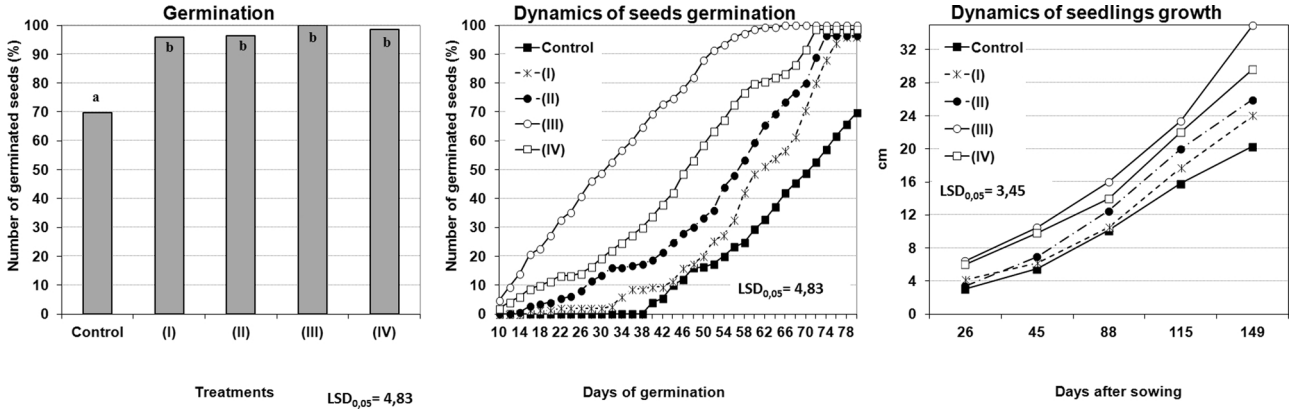

Fig. 5. The effect of treatment with heat shock selected phytohormones and compounds during seed stratification at 3 °C on percentage. dynamics of germination and growth of apple 'Ligol' plants. For the treatments, the mixture of compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (GA_3 + BAP + JA) was selected. The experiment was carried out in the following protocol:.

Control − untreated seeds.

Control – seeds stratified at 3 °C on the filter papers moistened with distilled water.

I. – 7 d seed stratification on filter paper moist. with H_2O → the exposure to heat shock (45 °C) for 2h → germination on the filter paper moist. with H_2O.

II. – 1 d seed soaking in compounds → stratification for 7 d on filter paper moist. with phytohormones → the exposure to heat shock (45 °C) for 2h → stratification on the filter paper moist. with phytohormones.

III. – 1 d seed soaking in compounds → stratification for 7 d on the filter paper moist. with phytohormones → incubation for 7 d on the filter paper moist. with phytohormones at 20 °C → stratification with phytohormones.

IV. – 1 d seed soaking in compounds → incubation for 7 d on the filter paper moist. with phytohormones at 20 °C → stratification with phytohormones. For explanation (I, II, III, IV) please refer to Materials and methods.

Table 6
The activity of acid (pH 6.0) and alkaline (pH 7.5) phosphatase, RNase and total dehydrogenases as affected by treatments with heat shock, selected phytohormones and compounds applied during seed stratification at 3 °C. Seeds treatments were carried out according to protocol shown in Fig. 5.

Treatments	Phosphatase (pH 6.0)	Phosphatase (pH 7.5)	RNase	Total dehydrogenases [mg(formazan) g^{-1}(seed FM)]
	[U g^{-1}(FM)]	[U g^{-1}(FM)]	[U g^{-1} (FM)]	
Control	0.28a	0.9a	1.50a	0,9a
I	0.32b	0.12b	1.94b	1,3b
II	0.35b	0.14b	2.34c	1,5b
III	0.47d	0.19d	2.78d	2,6d
IV	0.41c	0.16c	2.47c	2,1c
LSD 0.05	0.03	0.02	0.30	0,2

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

phytohormones the activity of acid (pH 6.0) and alkaline (pH 7.5) phosphatase, RNase and total dehydrogenases activity was positively affected (Table 6), which in turn contributed to the increased percentage, dynamics of seed germination and seedling growth as well as gas exchange and index of chlorophyll content (Table 7). Heat shock combined together with selected compounds and phytohormones (protocol II) additionally increased the examined parameters. However, stratifying of seeds in the presence of selected compounds and phytohormones, without subjecting them to heat shock exposure, were more beneficial.

3.6. The effect of pulsed radio frequency (PRF), selected phytohormones and compounds with a combination of stratification on apple seed germination, plant growth, and physiological activity

Further study was associated with the use of Pulsed Radio Frequency (PRF), which was incorporated into applied protocols. The demonstrated data clearly showed that PRF plays the additive role in breaking dormancy of apple 'Ligol' seed. PRF incorporated in the protocol I and III significantly increased percentage and dynamics of seed germination as well as dynamics of seedlings growth (Fig. 6). Further growth of seedlings maintained the beneficial effects of PFR application. After 149 days from sowing the seedlings derived from seeds treated according to protocol III (with PRF application plus compounds and phytohormones) were 4 cm taller than from protocol IV (without PRF exposure but with the application of compounds and phytohormones), and 12 cm higher than from control. Similarly, the seedlings developed from protocol I (with PRF application) were 3 cm taller that from protocol II (without PRF application), and 4 cm higher than control plants.

In respect of the activity of acid (pH 6.0) and alkaline (pH 7.5)

Table 7
Gas exchange and index of chlorophyll content in "Ligol" apple leaves as affected by compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (GA_3 + BAP + JA) applied during seed stratification at 3 °C. Seeds treatments were carried out according to protocol shown in Fig. 5.

Treatments	Net photosynthesis (μm CO_2 m^{-2} s^{-1}	Transpiration (nmol H_2O m^{-2} s^{-1})	Stomatal conductance (nmol H_2O^{-1} M^{-2} s^{-1})	Intercellular concentrate CO_2 (μmol CO_2 air mol^{-1})	Index of chlorophyll content in leaves
Control	1.72a	1.30a	159a	376e	27.9a
I	1,80ab	1,38b	174ab	348d	29,8a
II	1,88b	1,45c	195b	323c	33,7b
III	2,51d	1,71e	328d	263a	41,3d
IV	2,11c	1,57d	252c	298b	37,7c
LSD 0.05	0,09	0,06	35	24	3,1

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

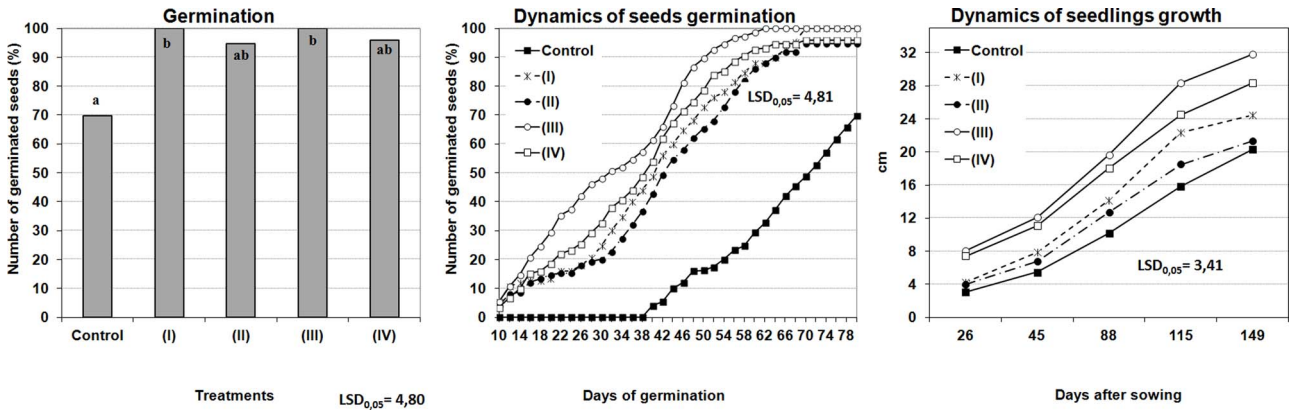

Fig. 6. The effect of Pulsed Radio Frequency (PRF), selected phytohormones and compounds applied during seed stratification at 3 °C on percentage, dynamics of germination and growth of apple 'Ligol' plants. For the treatments, the mixture of compounds (KNO_3 + Etephon + CO + H_2O_2) and phytohormones (GA_3 + BAP + JA) was selected. The experiment was carried in the following protocol:.
Control – seeds stratified at 3 °C on the filter papers moistened with distilled water.
I. – 1 d seed soaking in H_2O → the exposure for 1h to PRF 25 V/ 4 Hz/ 20 ms → stratification on the filter paper moist. with H_2O.
II. – 1 d seed soaking in H_2O → the stratification on the filter paper moist. with H_2O.
III – 1 d seed soaking in compounds → 7 days stratification in phytohormones → the exposure for 1 h to PRF 25 V/ 4 Hz/ 20 ms → stratification on the filter paper moist. with H_2O.
IV. 1 d seed soaking in compounds → 7 days stratification in phytohormones → stratification on the filter paper moist. with H_2O. For explanation (I, II, III, IV) please refer to Materials and methods.

Table 8
The activity of acid (pH 6.0) and alkaline (pH 7.5) phosphatase, RNase and dehydrogenases in apple embryos as affected by treatments with selected phytohormones. compounds and pulsed radio frequency (PRF) during seed stratification at 3 °C. Seeds treatments were carried out according to protocol shown in Fig. 6.

Treatments	Phosphatase (pH 6.0)	Phosphatase (pH 7.5)	RNase	Total dehydrogenases [mg(formazan) g^{-1}(seed FM)]
	[U g^{-1}(FM)]	[U g^{-1}(FM)]	[U g^{-1} (FM)]	
Control	0.28a	0.10a	1.50a	0,9a
I	0.39d	0.19d	2.48d	1,9d
II	0.31b	0.13b	1.84b	1,3b
III	0.45e	0.22e	2.82e	2,2e
IV	0.35c	0.16c	2.16c	1,6c
LSD 0.05	0.03	0.02	0.31	0,2

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

phosphatase, RNase and total dehydrogenases, the most pronounced results were obtained after seeds treatments according to protocol III i.e. application PRF combined with selected phytohormones and compounds (Table 8). Seeds subjected only to PFR exposure (without selected phytohormones and compounds in protocol I) also significantly promoted the activity of the examined parameters but to a lesser extent than following the protocol III. Physiological activity was also remarkably stimulated after application of protocol III (Table 9) suggesting that PRF plays the additive role in breaking dormancy of apple seeds.

4. Discussion

It is commonly considered that seeds extracted from apples are in a state of embryonic dormancy, which is a set of blocks imposed upon a process(es) essential for growth (Lewak, 2011) and is mainly expressed by inhibition of germination and abnormal seedlings (Gniazdowska et al., 2007). To remove the mentioned blocks and improve germinability the seeds are exposed to stratification (the influence of low temperature) for a required period. The presented results in Fig. 1 revealed that apple 'Ligol' seeds stratified (at 3 °C) in water germinated relatively slow with poor uniformity. Under the influence of stratification, the end of germination occurred only within 78 days at the level of 70% (Fig. 1). For the purpose of breeding programs, it is still not satisfactory result. Therefore, in the present research, the chosen methods are applied to verify the efficiency of breaking seed dormancy of apple 'Ligol'. The experiments were divided depending on applied phytohormones, compounds, and physical methods i.e. laser or red light, short-termed heat shock or Pulsed Radio Frequency (PRF) treatments.

The study with the separate use of KNO_3, ET, CO, H_2O_2 or applied together showed that the most beneficial effect was obtained due to stratification in the presence of KNO_3 (Fig. 1). Solutions of 0.1–0.2% KNO_3 is the most widely used chemical for promoting seed germination and are recommended by the Association of Official Seed Analysts and the International Seed Testing Association (Copeland and McDonald, 2001). It is applied for germination tests of many species and is also used to promote germination of some dormant seeds. However,

Table 9
Gas exchange and index of chlorophyll content in 'Ligol' apple plants. as affected by Pulsed Radio Frequency (PRF). selected phytohormones and compounds applied during seed stratification at 3 °C. Seeds treatments were carried out according to protocol shown in Fig. 6.

Treatments	Net photosynthesis (μm CO_2 m^{-2} s^{-1}	Transpiration (nmol H_2O m^{-2} s^{-1})	Stomatal conductance (nmol H_2O^{-1} M^{-2} s^{-1})	Intercellular concentrate CO_2 (μmol CO_2 air mol^{-1})	Index of chlorophyll content in leaves
Control	1.72a	1.30a	159a	356d	27.9a
I	2,18d	1,57d	223c	312b	48,4d
II	1,83b	1,42b	191b	331c	38,2 b
III	2,32e	1,68e	260d	281a	53,4e
IV	1,96c	1,50c	185b	311b	44,8c
LSD 0.05	0,11	0,06	29	18	3,3

*The data marked with the same letters within a column are not significantly different according to Duncan multiple range test at an alpha level of 0.05.

application of this compound is still not sufficiently explained regarding its mode of action (Çetinbaş and Koyuncu, 2006). Some studies demonstrated more stimulatory effects of KNO_3 in light than in darkness (Hilton, 1984). The others proposed that it contributed to stimulate oxygen uptake (Hilton and Thomas, 1986) or serves as a cofactor of phytochrome (Hilhorst, 1990). Nevertheless, KNO_3, similarly like in other studies, contributed to more effective stratification method and acted synergistically, or cooperatively with stratification and other compounds (Fig. 1).

The phytohormones (GA_3, BAP, JA) applied separately during stratification significantly accelerated dormancy release (Fig. 2, Tab 2). GA_3 has been found to be effective in breaking seed dormancy increasing in several species (Koyuncu, 2005). The balance between synthesis and catabolism of ABA and GAs as well as the sensitivity to these hormones are essential for dormancy status (Cembrowska-Lech and Kepczynski, 2016). Zhang and Lespinasse (1991) found that BAP stimulated even more efficiently germination of embryos excised from non-stratified apple seeds than GA_3. Ranjan and Lewak (1992) reported that jasmonic acid stimulated germination of seeds, which was accompanied by increased alkaline lipase. In another study, jasmonic acid also clearly stimulated germination of dormant apple seeds. However, in the seeds stratified for 30 and more days, some concentration of this hormone seemed inhibiting (Yildiz et al., 2007). However, SA applied separately revealed the inhibitory effect on all measured parameters (Fig. 2, Table 2). Apparently, this plant hormone did not play a pivotal role in the mechanism of dormancy removal of an apple seed. Application of SA, GA_3, BAP, JA simultaneously during stratification also significantly increased the percentage of seed germination. However, the selected plant hormones applied simultaneously in a mixture did not additionally increase percentage and dynamics of seed germination in comparison to GA_3 treatments. Presumably, some of the applied phytohormones inhibited another or appropriate concentration of each phytohormone applied in a mixture should be optimized. Lewak (2011) postulated that all hormones act collectively as a regulatory complex controlling the course of dormancy elimination. It seems that SA included in the mixture played the inhibitory effect in the regulatory complex since applied alone hindered the breaking of seed dormancy. It is possible that the levels of plant hormones should be monitored and supplemented at various phases of stratification. Lewak (2011) reported that there are three distinct phases of stratification course characterized by different changing the hormonal balance and around the 30th and 40th days of stratification a sharp rise in GAs, CKs, and JA levels took place reaching a maximum. It seems that the main obstacle is to determine the exact time and concentration of all plant hormones to break fully dormancy and accelerate this process. The present study also clearly demonstrated that stratification in the presence of GA_3 markedly accelerated further growth of plants (Fig. 2), which confirmed that it is a growth-promoting hormone involved in shoot growth (Linkies and Leubner-Metzger, 2012).

Further study with NO, HCL, laser and red light treatments showed that NO was the most effective compound in dormancy breakage (Fig. 3). Due to such treatment seed dormancy of apple 'Ligol' was completely removed (100% of seed germination) within 62 days and the obtained seedlings did not exhibit any morphological abnormalities characteristic for plants developed from dormant seeds. Grzesik and Romanowska-Duda (2015) concluded that the increased emission of NO by apple embryos during the early phase of germination is needed for the transition from dormant into the non-dormant state. Moreover, apple embryos pretreated with NO-induced transient accumulation of reactive oxygen species (ROS) leading to dormancy removal and germination. They also concluded that this gaseous stimulant accelerated the transition of germinated apple embryos from heterotrophy to autotrophy by stimulation of chloroplasts maturation (Krasuska et al., 2012). Apparently, demonstrated in the present study effects of NO application revealed these phenomena, which resulted in pronounced dormancy breakage and enhanced seedlings growth. SA was deliberately removed from the mixture of phytohormones applied in this protocol since previous results indicated that this plant hormone hindered dormancy removal.

The selected phytohormones and compounds were combined into protocols to arrange the most effective treatment for breaking seed dormancy of apple 'Ligol' (Fig. 4, Tables 4, 5). The most pronounced results were obtained (protocol III) when seeds stratified (at 3 °C) on a filter paper was moistened for 7 days with KNO_3 + Etephon + CO + H_2O_2 and then transferred on a filter paper moistened with GA_3 + BAP + JA till the end of seed germination. The application of this protocol could be a very useful tool in a shortening the apple breeding cycle, since the period of removing dormancy was reduced by 38 days in comparison to control (stratified in water) seeds.

Apple seeds subjected to 45 °C for 2 h (heat shock) positively affected dormancy alleviation but only to some degree, since combined with selected compounds and phytohormones rather inhibited their positive influences (Fig. 5, Tables 6, 7). Compounds and phytohormones appeared without heat shock affected more positively overcoming dormancy. Moreover, the presented data in Fig. 4 and Fig. 5 indicated that 1-day period of phytohormones or compounds inclusion during stratification is insufficient to assure a better balance of regulatory complex in apple seeds. As Lewak (2011) noted, the level of ABA decrease during stratification and disappeared about day 30th. However, the concentration of other phytohormones i.e. GAs and CKs, as well as that of JA, increased to reach a maximum level between the 30th and 50th days of stratification. Hence, the presence of selected phytohormones and compounds should be prolonged over 1-day during seeds stratification to allow them the absorb the needed for dormancy alleviation substances.

Interestingly, Pulsed Radio Frequency (PRF) incorporated into arranged protocols positively contributed to dormancy alleviation of apple seeds (Fig. 6, Tables 8, 9). PRF emitted by an RFG 3C PLUS lesion generator has been used in medicine in chronic pain therapy. Presumably, the PRF in the present study is used for the first time in order to alleviate seed dormancy. Their efficiency depends on the selected parameters: i.e. voltage (V), pulse repetition rate (pulses per second − Hz), and pulse duration (ms), and obviously a time of exposition. On the basis of preliminary experiment, the seeds were subjected to exposure of PRF for 1 h, at a constant voltage of 25 V, pulse repetition rates 4 Hz with pulse duration 20 ms. Nicholas at al. (2011) reported that biological changes in tissues during PRF can occur due to the thermal effects and the high intensity of electric fields, or as a result of both. However, in the present study, the chosen parameters did no generate any heat. Thus, it should be assumed that PRF generated only the high intensity of electric fields. Electric fields can have plausibly significant effects on cells because of the transmembrane potentials that they induce (Chua et al., 2011). It can be assumed from the present study that PRF also contributed to dormancy removal since it is clearly visible that applied alone during stratification (protocol I) or together with selected phytohormones and compounds (protocol III) increased germinability of seeds as well as dynamics of seeds germination and growth of normally developed seedlings.

The conducted experiments indicated that application of the applied compounds, phytohormones, heat shock and PRF to apple seeds stratified at 3 °C explicitly increased the activity of acid and alkaline phosphatase, RNase, and total dehydrogenases, which play an important role during seed germination and plant development (Tables 1–9). The alkaline and acid phosphatase is responsible for the distribution of phosphorus in seeds and plants and they catalyze the hydrolysis of organic phosphorus. They are also considered to be a good indicator of the activity of secondary metabolites released from the environment in which seeds are germinated and they show the mineralization potential of organic phosphorus or biological activity of soil (Dick and Tabatabai, 1993).

The studied compounds, hormones and PRF applications to apple seeds stimulated also ribonuclease (RNase) activity. RNase constitutes a

heterogeneous group of enzymes involved in the process of enzymatic degradation of various fractions of ribonucleic acid. Their activity increases during apoptosis, aging of plants and seed germination. Booker (2004) indicated that ribonuclease modified the activity of particular genes by the specific degradation of mRNA transcripts leading to changes in the concentration of molecules of these compounds. According to Lehmann et al. (2001), Sindelarova et al. (2005) and Srivastava et al. (2006) RNase activity can increase, among others, after the attack of phytopathogens and under phosphorus deficiency conditions. Stimulation of RNase activity may play an important role in increasing defense mechanisms in seed and plant tissues, as it was also observed in willow plants, as well as in corn grains and plants, in which the improved health status was associated with the enhanced RNase activity (Grzesik et al., 2017; Grzesik and Romanowska-Duda, 2015).

The demonstrated data showed the possibility to overcome apple 'Ligol' seed dormancy more effectively by improved stratification than by commonly applied stratification in water at 3 °C. As presented Lewak (2011) most of the applied compounds and phytohormones positively affected dormancy elimination. They can act collectively as a regulatory complex controlling the course of dormancy removal. Physical methods (PRF and heat shock) additionally contributed to dormancy breakage. As was observed with heat shock treatments, the influence of phytohormones or compounds only for 1-day seems to be a too short period to optimize stratification process. Time of phytohormones or compounds impacts should be prolonged to minimum 7 days to assure more balanced conditions for shortening the period of dormancy removal. Further research is needed to optimize stratification methods with appropriate contents and concentration of compounds and phytohormones combined with PRF exposure. The elaborated methods of improved stratification can be very useful in plant breeding by shortening the breeding of new apple varieties.

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