Effects of Brassinosteroid Application on Seed Germination of Scots Pine under Standard and Heat Stress Conditions

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Abstract

We assessed the effect of brassinosteroid 2α, 3α, 17β-trihydroxy-5α-androstan-6-one on seed germination of *Pinus sylvestris* L. Brassinosteroids are phytohormones that play a key role in plant growth and affect a substantial part of their physiological processes.

A total of 6,400 seeds were used in the experiment, divided into 8 groups based on a combination of temperature stress and concentration of the applied brassinosteroid solution in which the samples were soaked before testing. The four brassinosteroid treatments in order of their concentrations were as follows: control (0 mg/l), low (0.004 mg/l), medium (0.04 mg/l) and high (0.4 mg/l), combined with either stress temperature regime, where the seeds were temporarily exposed to high temperature up to 39 °C, or normal temperature regime without any heat stress. Germination capacity and the length of radicles were evaluated weekly.

The results showed a positive effect on seed germination under standard regime for low brassinosteroid concentration (0.004 mg/l), while no significant effect was recorded for seeds treated with medium (0.04 mg/l) and high (0.4 mg/l) brassinosteroid concentration The application of convenient brassinosteroid concentrations reduced the negative impact of heat stress and improved the germination ratio of seeds that were not heat-stressed.

Keywords: germination capacity, germination energy, brassinosteroid, high temperature stress.

Introduction

A number of studies aimed at the support of seedling growth on various forest sites can be found in forestry literature (Erefur et al. 2008, Cukor et al. 2017, Pokharel et al. 2017), however, less attention has been paid to stimulation of germination that is influenced by environmental and hormonal factors. One possibility to support seed germination of forest species is the application of brassinosteroids, where the positive effect on germination has been proved in several studies on agricultural crops seeds (Anuradha and Rao 2007, Vardhini and Rao 2003, Anduradha and Rao 2003, Kartal et al. 2009). Preliminary experiments using a brassinosteroid application to encourage seed germination in tree species were documented on *Robinia pseudoacacia* L., *Pinus tabulaeformis* Carr. and *Ailanthus altissima* Mill. by Li et al. (2002, 2005)

Brassinosteroids are a group of phytohormones (Bishop and Yokota 2001, Sharma et al. 2017), representing the polyhydroxysteroid class. Currently, several dozens of brassinosteroids have already been described in many plant species (Yokota 1997, Clouse and Sasse 1998, Steber and McCourt 2001, Bajguz and Hayat 2009, Kutschera and Wang 2012). Brassinosteroids play a crucial role in plant development as they influence growth and plant physiology (Sasse 1997, Vriet et al. 1998, Anduradha and Rao 2003). Previous research on the effects of brassinosteroids on plants have shown that the hormones affect a variety of physiological responses such as stem and leaf lengthening, bending and unfolding of leaves, pollen tube growth, induction of ethylene biosynthesis (Salchert et al. 1998, Dhaubhadel et al. 1999, Bajguz 2007, Zhang et al. 2014), development of blooms and fruits, initiation of the flowering process, activation of the proton pump and activation of photosynthesis (Zhang et al. 2005, Bajguz and Hayat 2009).

Grove et al. (1979) first isolated brassinosteroid (brassinolide) from Rapeseed (*Brassica napus* L.) and Yokota et al. (1982) isolated the precursor of brassinosteroid from insect galls on Japanese Chestnut (*Castanea crenata* Siebold & Zucc.). The occurrence of low concentrations of brassinosteroids was documented across the plant kingdom in various plant organs (Fujioka and Sakurai 1997) and it was found that plant tissues of young individuals contain higher levels of brassinosteroids compared to adults; the richest source being pollen and immature seeds, where the detected values are ranging from 1-100 ng per gram of fresh material (Rao et al. 2002).

Extremely high temperatures and water deficiency belong among the most important abiotic stressors that limit the plant growth and impair its viability (Vardhini and Rao 2003, Allakhverdiev et al. 2008, Wu et al. 2011). It was reported (Bajguz and Hayat 2009, Vriet et al. 2012, Fariduddin et al. 2014, Ali 2017) that the effects of these stressors can be mitigated by brassinosteroids.

Heat stress can occur also in nurseries of temperate zone. The surface of seed beds can become very hot even in spring during bright days (McDonald 1984) and the process of germination can be worsened significantly if artificial irrigation enabling cooling sprays is absent. Tree species with small seeds, whose sowing depth is limited, are most vulnerable.

Recently, the effects of brassinosteroids on mortality, height growth and chlorophyll fluorescence in foliage of seedlings of selected tree species under forest nursery conditions have been tested e.g. by Nováková et al. (2015). Their impact on germination and extenuation of drought stress for some tree species was evaluated by Kune□ et al. (2017).

In this paper, the effect of temperature regime and synthetic brassinosteroid applied at various concentrations on Scots pine (*Pinus sylvestris* L.) seeds were assessed under controlled conditions of a growth chamber. The aims of the study were (1) to evaluate how various concentrations of the selected brassinosteroid compound influence the germination capacity of seeds exposed to different temperature regimes and (2) to describe the impact of temperature stress on the development of germination percentage.

Materials and Methods

In this experiment, brassinosteroid 2α, 3α, 17β-trihydroxy-5α-androstan-6-one was manufactured by PHPchem Ltd. (Neratovice, Czech Republic) for testing on Scots pine seed germination. The experiment started on 12 June, 2015, in the Seed Laboratory of the Department of Silviculture, Faculty of Forestry and Wood Sciences, Czech University of Life Sciences.

The seed lot (code number CZ-2-2B-BO-3023-18-2-L) used in the experiment originated from the North Bohemian Sandstone Plateau and the Český ráj sandstone area, Beech-Oak forest vegetation zone (*Fageto-Quercetum*) (Viewegh et al. 2003) with an average annual temperature of 7.3–8.5 °C and an average annual precipitation of 550–724 mm (Plķva 1987, Mikeska and Prausová 2013). The seeds used in our study matured in 2005; the weight of 1,000 pure seeds was 6.370 g, and seed purity was 98.5%. The seed lot was long-term stored in vacuum at 1 °C until June 2014, then transferred to plastic bags and stored at 6–7 °C until the experiment began. Except for soaking, the seeds were not pretreated before sowing.

A total of 6,400 seeds were used in the experiment and split into eight groups of 800 seeds each for different combinations consisting of a particular brassinosteroid treatment and a particular temperature regime.

The brassinosteroid treatments were prepared by first dividing the seeds into four equal groups of 1,600. Each group was sterilized in 1% sodium hypochlorite solution for 2 minutes and then thoroughly rinsed in distilled water. After sterilization and rinsing, the seed groups were split and imbibed in four solutions of brassinosteroid (0.004, 0.04 and 0.4 mg/l respectively) or in distilled water as the control treatment for 48 hours at a temperature of 20 °C. The original concentration of 0.4 mg/l was chosen because of the best recorded germination capacity in study by Li et al. (2005), other tested concentrations were selected as one and two decimal order lower values.

One half of each group (treatment) was germinated under a normal (non-stressed) regime and the other half with an induced temporary heat stress. The test scheme and the concentrations of brassinosteroid in the treatments are shown in Table 1.

Seeds were germinated on sterile water-soaked filter paper 80 g/m^2 placed in capped transparent plastic

Table 1. Description of combinations of used treatment and stress regime, design of experiment

Treatment	Germination regime	Treatment-Regime Combination	Treatment-Regime code	Total number of seeds
Without brassinosteroids	Normal	Control non-stressed	C	800 (8×100)
0.004 mg/l	Normal	Low non-stressed	L	800 (8×100)
0.04 mg/l	Normal	Medium non-stressed	M	800 (8×100)
0.4 mg/l	Normal	High non-stressed	H	800 (8×100)
Without brassinosteroids	Stress	Control stressed	CS	800 (8×100)
0.004 mg/l	Stress	Low stressed	LS	800 (8×100)
0.04 mg/l	Stress	Medium stressed	MS	800 (8×100)
0.4 mg/l	Stress	High stressed	HS	800 (8×100)

boxes (6.5×9×3.5 cm) arranged on shelves of the growth chamber in Latin square design. The seeds were put on the paper in the boxes (100 seeds per box) not touching each other. They were gently sprayed with distilled water and the boxes were capped before being placed in the growth chamber. The boxes represented replications of the treatment-regime combinations.

In the non-stressed regime, germination was conducted in an incubator with alternating temperatures of 20/30 °C (dark/light) with eight hours of fluorescent illumination (approximately 11,000 lux, colour temperature of 4,000 K) per day.

In the heat-stressed regime, on the sixth day after the start of the germination process, the boxes with seeds designated for exposure to the heat stress were transferred to another growth chamber (in Latin square arrangement) where a heat stress programme lasting 10 hours was launched with temperatures culminating at 39 °C. The temperature-time profile of the simulated stress is depicted in Figure 1. After the simulated temporary stress, the samples were transferred back and germination continued under the conditions of the normal (non-stressed) regime.

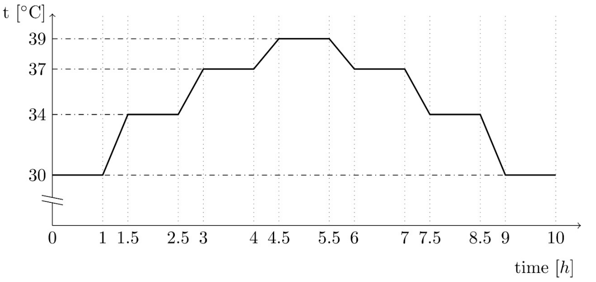

Figure 1. Temperature-time profile of the simulated stress included in the germination stress regime on the 6th day after the start of germination

Germinants were counted weekly throughout the germination test for 28 days with the first measurement of germinated seeds occurring on the seventh day of the experiment. Seeds whose radicles were minimum four times the seed length were counted as germinated. The germinated seeds were removed from the germination box and the length of their radicles was measured to the nearest 1 mm. The same evaluations followed on the 14th, 21st and 28th day of the experiment and the medium was moistened with distilled water when necessary.

Statistical evaluation of germination percentage was carried out using the chi-square test of independence in contingency table with multiple comparisons of quantities with binomial distribution (Agresti et al. 2008) and the lengths of the radicles were compared using the Kruskal-Wallis test followed by multiple comparisons. The evaluation was done in the R statistical software, version 3.1.2 (R Core Team 2017). All hypotheses were tested at a significance level of α=0.05.

Results

Evaluation of the effect of brassinosteroids on germination

The lowest germination capacity values were recorded for the combination of stress regime with a zero dose of brassinosteroid – CS (46.0%) and a stress regime with a low dose of brassinosteroid – LS (51.8%). The highest germination capacity was found in low-dose brassinosteroid treated – L (76.0%) seeds. Results of germination tests are shown in Figure 2. The pattern of response to decreasing concentration of brassinosteroids for heat-stressed seeds is different in comparison with those of a non-stressed regime. While medium concentration of brassinosteroid had no positive effect on seed germination for standard regime and higher rates of fully germinated seeds were observed for low and high brassinosteroid concentration, the ratio of fully germinated seeds under stress regime increased with increasing brassinosteroid concentration.

The chi-square test of independence showed highly significant effects (chi-squared=229.04, *df*=7, $p < 0.001$) of treatment-regime combination on germination capacity.

Subsequent multiple comparisons showed that under the heat-stress regime, no significant differences were noticed between untreated seeds (CS) and those treated with low concentration of brassinosteroid (LS). A significantly higher germination capacity compared to CS and LS was found in seeds treated with a solution of medium (MS) and high (HS) brassinosteroid concentration. The greatest effectiveness was found in the high concentration (HS) treatment.

Under the standard regime, germination capacity of untreated seeds (C) was not statistically different from those treated with medium (M) or high (H) concentration of brassinosteroids. The highest germination capacity was recorded for low concentration (L) of brassinosteroids, which was significantly higher compared to control variant (C) but non-significantly higher compared to variant with high concentration of brassinosteroids (H).

Statistical evaluation also revealed that seeds exposed to heat stress, when treated with medium (MS) or a high concentration of the brassinosteroid (HS) solution, had comparable germination capacity as unstressed control samples germinated under standard conditions (C), 62.4% for HS variant and 65.4% for unstressed control samples, respectively.

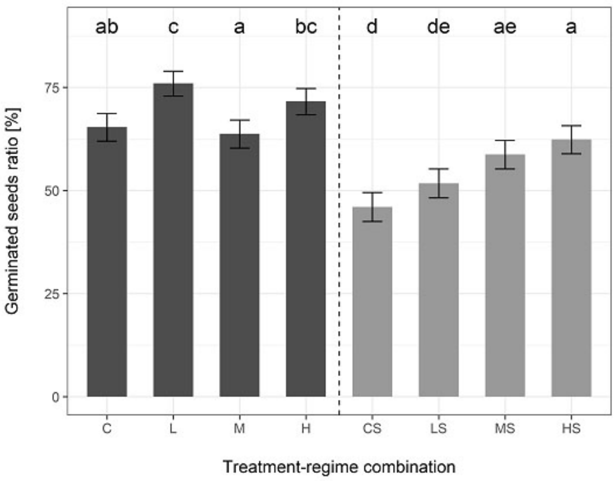

Figure 2. Germination capacity (proportion of germinated seeds) after the experiment termination (28th day) for particular combinations of treatment and germination regime, see Table 1. Dark gray columns denote normal regime, light gray columns denote stress regime. Statistically significant differences are distinguished by letters displayed above columns. The error bars depict the 95% confidence intervals

Development of germination capacity in weekly intervals

Radicle lengths were measured each week and the mean radicle lengths altogether with results of statistical testing of their differences are depicted in Table 2.

After the first week, the highest number of germinated seeds (103 seeds) was found in combination of low brassinosteroid concentration under a normal temperature regime. The shortest mean radicle length (20.0 mm) was recorded among the heat-stressed seeds treated with low brassinosteroid concentration treatments, the longest one was recorded for control variant of heat-stressed seeds (26.1 mm), see Table 2.

Table 2. Numbers and mean radicle lengths of completely germinated seeds of *Pinus sylvestris* L. throughout the experiment (at weekly intervals). The Kruskal-Wallis test with subsequent multiple comparisons were performed to distinguish statistically significant differences between selected variants for each week

Treatment	First week		Second week		Third week		Fourth week	
	Count	Mean, mm	Count	Mean, mm	Count	Mean, mm	Count	Mean, mm
C	66	23.7 ab	287	36.6 c	130	30.2 a	40	20.2 ac
L	103	21.7 a	405	44.2 d	73	23.5 b	27	29.8 b
M	55	27.1 b	271	33.0 abc	168	31.5 a	16	23.6 abc
H	47	24.0 ab	316	31.3 ab	189	34.0 a	21	21.9 abc
CS	93	26.1 b	217	34.3 bc	32	23.8 b	26	20.6 abc
LS	73	20.0 a	168	44.3 d	121	24.9 b	52	21.3 ac
MS	15	21.6 ab	183	32.8 abc	197	25.7 b	75	19.9 c
HS	48	20.8 a	195	30.3 a	203	32.3 a	53	28.0 ab

Note: Different characters following the mean values indicate significant differences at significance level $\alpha = 0.05$.

Under the normal regime, the highest number of seeds germinated during the second week; the low, L (405 seeds), and high, H (316 seeds), concentrations acted most efficiently. Under the stressed regime the results differed in the second week from the normal regime. Most seeds germinated in the control treatment, CS (217 seeds), however, the germination percentage strongly declined, which affected the total germination capacity of this treatment regime combination. The highest mean value of radicle length was recorded for the treatments with a low concentration of brassinosteroids for both non-stressed, L (44.2 mm) and stressed, LS (44.3 mm) samples.

In the third week, all variants germinating under the normal regime showed a significant decrease in numbers of germination, while the brassinosteroid treatments under the stress regime did not manifest similar results until the fourth week. The third and fourth week under the stress regime, the treatments with brassinosteroid application showed significantly higher numbers compared to the relevant stressed control.

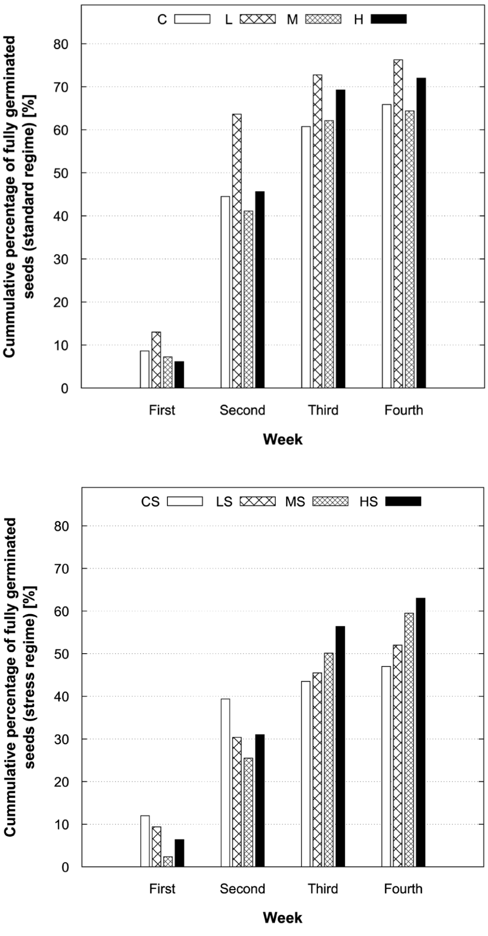

Figure 3. Development of germinated seeds proportion during the experiment in individual combinations of treatments and temperature regime

Discussion

Current trend of global climate change indicates that temperature stress will most likely be of increasing importance not only in agriculture (Shah et al. 2011) but also in forestry. Temperatures in Europe are likely to increase more than the global mean (Hansen et al. 2010) as evidenced by Europe experiencing unprecedented heat waves during the summers of 2003 or 2010 (EASAC 2013). Effects of within one month temperature anomalies are significantly greater than seasonal anomalies and effects of daily weather fluctuations are much larger than effects of global warming (Hansen et al. 2010). In comparison to air temperature (recorded in shade), the temperature extremes on bare soil surfaces of seedbeds in forest nurseries are markedly more pronounced (Stoeckeler and Slabaugh 1965). Artificial irrigation and shading of seedbeds should be the principle measures to protect the germinating seeds and emerging seedlings in the nurseries from heat stress. Nonetheless, a search for additional ways to decrease the vulnerability of germinating seeds and seedlings is desirable and brassinosteroids could have a potential in this field.

Germination is a highly vulnerable developmental period of each plant (Angevine and Chabot 1979) and Scots pine, a pioneer species, is not excluded as the covering depth of its seeds is relatively small (ca 8 mm) and seedbed surfaces can become hot.

The aim of this study was to evaluate the effect of three different concentrations of brassinosteroid (2α, 3α, 17β-trihydroxy-5α-androstan-6-one) on germination of Scots pine seeds under standard and heat stress regime. A positive effect on seed germination was presumed as it was previously documented on a wide range of plants (Sharma et al. 2008, Ahammed et al. 2012), most thoroughly the influence of brassinosteroids was described in agricultural crops. Recently, Doležalová et al. (2016) confirmed a positive effect of brassinosteroids on the size of tubers and overall onion (*Allium cepa* L.) yield. The effect of providing increased yield was previously recorded in other crops, such as *Oryza sativa* L., *Solanum tuberosum* L., *Zea mays* L. or *Sinapis alba* L. (Khripach et al. 2000, Zullo and Adam 2002, Holá et al. 2010, Janeczko et al. 2010). A higher quality yield was also documented for agricultural crops treated with brassinosteroids (Vardhini and Rao 1998, Holá et al. 2010, Koudelka et al. 2012, Liu et al. 2017). Brassinosteroids may also induce stalk extensions (Pereira-Netto et al. 2006, Bajguz 2007). Accelerated germination and faster growth of the primary root were witnessed in barley (*Hordeum vulgare* L.) (Kartal et al. 2009). The effect of various concentrations of these phytohormones on the length of primary roots was also documented for the *Arabidopsis* genus (Steber and McCourt 2001, Haubrick and Assmann 2006, Kim et al. 2007). The application of brassinosteroids can also influence tolerance to diverse types of stress. In the primary stage of growth, a positive effect on salt stress reduction was determined in rice (*Oryza sativa* L.) (Anuradha and Rao 2003, Nunez et al. 2003) and other agricultural plants (Shahbaz et al. 2008, Shahid et al. 2011).

With respect to our results, the positive effect of brassinosteroids was also described in response to temperature stress. Brassinosteroids were, for example, proven to mitigate the effect of stress on germination of seeds of *Cicer arietinum* L. exposed to a low temperature of 0°C (Hayat et al. 2014). The effect of brassinosteroids was described in *Lycopersicon esculentum* Mill., where the brassinosteroid treated plants had significantly better fruit production compared to those untreated after low-temperature stress (Khan et al. 2015).

In our experiment, a positive response to phytohormone application was found in a combination of a standard temperature regime and low and high brassinosteroid concentrations; the medium dose acted unfavourably. Different trend was observed for variants exposed to heat stress, where the brassinosteroid effect positively correlated with its concentration. It can be seen that brassinosteroids are able to maintain a comparable overall germination capacity of stressed seeds at appropriate concentrations in comparison with the non-stressed control combination but are unable to prevent decreasing germination energy. Similar results were observed by Kuneš et al. (2017), where brassinosteroid treatment (0.1 mg/l) had stronger effect on germination capacity in low humidity stress than in seeds germinating under standard conditions in optimal humidity.

The absence of a positive effect of brassinosteroid applied at medium concentration remains an unresolved question as both lower and higher concentrations triggered a positive effect under non-stressed germination regime. The seed boxes of the compared concentrations and controls in the growth chamber were evenly distributed in the individual sections. A similar phenomenon was also observed in the study by Morales (2015) for hypocotyl and root lengths of *Lactuca sativa* L. treated with leachate of *Beilschmiedia tawa* (A. Cunn.) Kirk. This observation brings a question of appropriate concentration.

Kuneš et al. (2017) recorded reduced germination percentage of Scots pine seeds treated with 0.1 mg/l brassinosteroid (2α, 3α, 17β-trihydroxy-5α-androstan-6-one) concentration under optimal conditions. It is of note that 0.1 mg/l and 0.04 mg/l (our medium concentration) are placed between 0.4 mg/l and 0.004 mg/l concentrations that acted positively in our study, this would suggest a potential of causality. Objectively, any hidden problem that is not related to the suitability of the medium concentration of the hormone but methodology should not be excluded at the moment (undesirable contamination of the medium concentration solution, insufficient mixing). Our yet unpublished data on European Black Pine (*Pinus nigra* Arnold) did not show retardation when treated with 0.04 mg/l solution of 2α, 3α,17β-trihydroxy-5α-androstan-6-one and had responded positively, however, the responses of two various species do not have to be consistent.

Li et al. (2005) recommended 0.4 mg/l of natural brassinolide for Tree of Heaven (*Ailanthus altissima* Mill.) and in contrast to the results presented in this study, the authors observed a consistent increase in positive reaction with rising brassinolide concentration until optimal concentration (0.4 mg/L) was achieved.

According to the aforementioned research, to assess 2α, 3α, 17β-trihydroxy-5α-androstan-6-one for various trees attention should be focused on concentration and choosing the right concentration of brassinosteroid solution as mentioned by Gomes (2011). However, testing the influence of various brassinosteroid compounds and their optimal concentrations for various forest tree species is relatively new and further research is required.

Conclusions

The application of brassinosteroid 2α, 3α, 17β-trihydroxy-5α-androstan-6-one represents a potential method of preventing the reduction in germination capacity of Scots pine (*Pinus sylvestris* L.) seeds due to temporary stress caused by elevated temperature. The results of this study showed that application of a suitable concentration of the tested hormone on the pine seeds may improve germination capacity and partially compensate for the negative effect of temporary heat stress. It was found that for seeds experiencing temporary stress due to elevated temperature, a seed treatment with low and high brassinosteroid concentrations helped to maintain germination capacity at values comparable to a non-stressed control. However, brassinosteroid application did not prevent the decrease in germination energy due to simulated stress. Our results also indicate the importance of a suitable concentration of brassinosteroids.

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