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USE OF BRASSINOSTEROIDS TO OVERCOME UNFAVORABLE CLIMATIC EFFECTS ON SEED GERMINATION OF CHOSEN TREE SPECIES FROM TEMPERATE FORESTS

Diploma Thesis
In Forestry, Water and Landscape Management

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Prague 2015

Statutory declaration

I hereby certify that I have elaborated my thesis independently, only with the expert guidance of my thesis director Ing. Ivan Kuneš, Ph.D.

I further declare that all data and information I have used in my thesis are stated in the references.

In Prague …….. ……

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Acknowledgements

First of all I would like to express my heartfelt gratitude to my supervisor Ing. Ivan Kuneš, Ph.D., for making the facilities available to carry out this study and his supervision and excellent guidance throughout the study.

My deepest gratitude is due to Prof. Ing. Vilém Podrázský CSc. Head, Department of Silviculture, Czech University of Life Sciences Prague, for giving me an opportunity to carry out this study.

I would like to appreciate the invaluable assistance given by Ing. Olga Nováková, Ph.D., Ing. Martin Baláš, Ing. Nada Rašáková, and Bc. Josef Gallo, for their help during this study period.

My sincere thanks are extended to Bc. Martin Havlíček, Bc. Vasco Valério, Bc. Lukáš Koubek, Bc. Marie Netopilová, Sujeewa Buddasiri and Zhansaya Bolatova for their encouragement and invaluable help during my study period and stay in Czech Republic.

I would like to express my special thanks and heartfelt gratitude to my Mother, Dissarathna Menike Dissanayaka, my father P.A.Suraweera and my lovely wife Geethanjali Athukorala and children for the unconditional support as I've found far from my home.

Abstract

Many seeds have an ability to overcome some adverse climatic conditions, however many forest seeds from temperate regions come across with some germination and survival problems when they are exposed to adverse climatic conditions of the environment during their germination period. Brassinosteroids (BRs) regulate several kinds of plant activities and they can help to overcome these adverse climatic conditions. The effects of exogenous application of BRs on germination capacity and germination energy of European black pine (*Pinus nigra*) seeds were tested in this study.

For this study a synthetically prepared analogue of 24-epibrassinolid (brassinosteroid-2α,3α,17β-trihydroxy-5α-androstan-6-one) was used. Four different concentrations of BRs and control were used under two regimes: optimal conditions and stress conditions which means that seeds were exposed to high temperatures up to 42°C. Seeds were soaked for 24 hours. After that seeds were planted in containers and placed into growth chambers. Fully germinated seeds were counted and radicular length of each seed was measured.

In the case of the optimal conditions results of germination capacity showed significant difference between control and high concentrations of BRs treated seeds. Under the stress conditions there was observed significant difference between control and medium BRs concentration. In the case of germination energy there was also a significant difference between control and medium concentrations of BRs treatments. In this study was found out that the BRs improve the germination capacity and germination energy of *Pinus nigra* seeds under the high temperatures.

Key words: Brassinosteroids, *Pinus nigra*, germination capacity, germination energy

Abstrakt

Mnoho semen má schopnost překonat některé nepříznivé klimatické podmínky, nicméně mnoho semen z lesů mírného pásma může mít určité problémy s klíčením a také s přežitím, pokud jsou tato semena během svého klíčení vystavěna těmto nepříznivým klimatickým podmínkám. Brassinosteroidy (BR) regulují některé rostlinné aktivity a můžou pomoci tyto nepříznivé klimatické podmínky překonat. V této studii byly testovány účinky exogenní aplikace brassinosteroidů na schopnost klíčení a na energii klíčení borovice černé (*Pinus nigra*).

Pro tuto studii byla použita synteticky připravená obdoba 24-epibrassinolidu (brassinosteroid-2α, 3α, 17β-trihydroxy-5α-androstan-6-one). Čtyři různé koncentrace BR a tzv. kontrol byly použity ve dvou režimech: v optimálních podmínkách a také ve stresových podmínkách, což znamená, že semena byla vystavena vysokým teplotám až 42 °C. Semena byla namočena po dobu 24 hodin a poté byla vysazena do kontejnerů a umístěna do růstových komor. Zcela vyklíčená semena byla spočítána a délka kořene každého semene byla změřena.

V případě schopnosti klíčení semen rostoucích za optimálních podmínek výsledky ukázaly významný rozdíl mezi kontrolem a semeny ošetřenými vysokou koncentrací BR. Ve stresových podmínkách byl pozorován významný rozdíl mezi kontrolem a středními koncentracemi BR. V případě energie klíčení byl také pozorován významný rozdíl mezi kontrolem a střední koncentrací BR. V této studii bylo zjištěno, že BR zlepšují schopnost klíčení a energii klíčení semen *Pinus nigra* za vysokých teplot.

Klíčová slova: Brassinosteroidy, *Pinus nigra,* schopnost klíčení, energie klíčení

List of Abbreviations

°C - Celsius/Centigrade

°F - Fahrenheit

ABA - Abscisic acid

BR's - Brassinosteroids

C - Controlled/ Water socked only

EBR - 24-epibrassinolide

GA - Gibberellins/ Gibberellic acid

H - High concentration

HSPs - Heat shock proteins

i.e. - That is; in other words; that is to say.

IAA - Indole-3-acetic acid

ISTA - International Seed Testing Association

L - Low concentration

lx - Lux

M - Medium concentration

Mc - Medium-Channa concentration

mg/l - milligrams per litter

NaCl - Sodium chloride/ Salt

NBR - Natural brassinolide

O - Optimal environmental conditions

p - Probability

pH - Negative logarithm of the hydrogen ion concentration/ - log[H^+]

RNA - Ribonucleic acid

S - Stress conditions

SE - Somatic embryogenesis

vs - Versus

WGA - Wheat germ agglutinin

ZR - Zeatin riboside

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1. Introduction

1.1 General over view

Seed can be defined as a basic reproductive structure of plants that consists of a plant embryo to produce a new plant. Most of Gymnosperms and Angiosperms plants produce seeds for their progeny survival (Bonner, 2008).

Many of the forest trees are usually propagated by seeds. Those seeds are usually tiny and some of them can have an ability to remain viable for long time period without germinating (Bewley & Black, 1995). However, the longevity of seeds varies widely: some seeds remain viable for only about few days and others have been known to germinate after hundreds or even thousands of years (Roach, 2005; Kaufman, 2012). Many forest seeds encounter with seed dormancy and have an ability to overcome the adverse climatic conditions. Seed dormancy means that even though the viable seed meets with favorable climatic conditions it is unable to germinate due to some kind of inherence within the seed such as plant hormones (Bewley, 1997). Plant hormones act on seeds in positive or inhibiting way of their germination process.

Brassinosteroids (BRs) are a group of phytohormones which regulate several kinds of plant activities such as fruit bearing, seed germination, and ability to overcome adverse conditions of plant (Bajguz & Shamsul, 2009). Many forest seeds from the temperate regions face more variations of climatic conditions than the seeds from the tropics. This research has been done to find out the BRs' effects on seed germination on European black pine (*Pinus nigra*).

1.2 Objectives

The main objective of the study is to assess whether the BRs can:

- improve the germination capacity and germination energy of seeds
- BRs can alleviate the stress of seed in the course of germination.

2. Literature Review

2.1. Seeds

Seed can be define as a mature fertilized ovule of angiosperms and gymnosperms that contains an embryo and the nutrition reserve it will need to grow into a new plant. Seeds provide a great reproductive advantage in being able to survive for extended periods until environment conditions are favorable for germination and growth (Bewley & Black, 1995).

Under the plant kingdom, the seed producing organisms belong to the division Spermatophyta and are further classified into 2 sub-divisions i.e Gymnospermae (gymnosperms) and Angiospermae (angiosperms). Within this two sub-divisions majority of forest plants belong to the order Coniferales (conifers) under the sub-division Gymnospermae. Pinaceae, Taxodiaceae, Cupressaceae, and Taxaceae are the most economically important families and pine (*Pinus* L.), spruce (*Picea* A. Dietr.), sequoia (*Sequoia* Endl.), cypress (*Cupressus* L.), and yew (*Taxus* L.) are the genera which having the heights demand in forestry (Bonner, 2008).

The mature gymnosperm seed consists of the following parts. (when seed matured some parts disappear in some species):

1. The seed coat or testa developed from the integument, its diploid from the female parent.

2. The diploid perisperm, developed from the nucellus. In most gymnosperm species this is absorbed by the female gametophyte and has disappeared by the time the seed is well matured or ripe, but it is still recognizable as a distinct tissue in a species like *Pinus pinea* (Willan, 1985).

3. The haploid female gametophytic tissue. This serves as a food storage organ to nourish the embryo. It is similar in function in the endosperm of angiosperms.

4. The embryo, with the same parts appears in angiosperms, radicle, cotyledons, plumule and hypocotyl. Unlike in angiosperms the number of cotyledons varies between and within genera, some *Pinus* species being up to 18 (Bonner, 2008), compared with the constant two in the dicotyledons which comprise the great majority of angiosperm trees. As same as in all

angiosperm seeds the essential constituents of embryo, protective covering and food storage tissue are present in all gymnosperm seeds too (Willan, 1985; Bonner, 2008).

2.1.1. Forest seeds

There is a wide variation in the interval between flowering and seed maturity and dispersal. This happens due to the lengthy gap between pollination and fertilization of several gymnosperm species. Total period between pollination and cone maturity is usually about two years in many species of the genus *Pinus* (Willan, 1985). In several temperate species such as *Pseudotsuga menziesii* the development is completed within 5 month, a single season (Allen & Owens, 1972).

After fertilization the female cone increases in size and weight, in moisture content and accumulated nutrition reserves. When the cone comes to maturity, the moisture content decreases and accumulated food reserves move from cone to seed and the cone becomes more or less woody. However the woody cone is the most characteristic type of fruit in gymnosperms (Willan, 1985; Bonner, 2008). Parthenocarpy is rare in pines but common in *Abies*, *Juniperus*, *Larix*, *Picea*, *Taxus* and *Thuja* (Kozlowski, 1971).

2.1.1.1. European black pine (*Pinus nigra*)

European black pine (Austrian pine) having dark green foliage, distinctive form, and it is adapted to a wide range of soil types and shows widespread distribution; its discontinuous natural range extends from Morocco, eastern Spain, and Algeria in western Europe to southern and eastern Turkey; and in the north from Austria and former Yugoslavia to the Crimea, Russia (Critchfield & Little, 1966). The species encompasses a host of recognized varieties and cultivars. The Corsican variety has notably better quality of wood than typical Austrian pine. The var. caramanica, Rehder in Cyprus, Turkey, and the Crimea general tends to have the largest seeds, 38,500 to 45,760 seeds per kg; while var. Corsicana in Corsica has the smallest seeds, 61,600 to 79,000 seeds per kg (Krugman & Jenkinson, 2008). In Belgium are believed to represent one of the more cold-hardy varieties (Wheeler, et al., 1976). Physiological traits draw up the boundaries of 3 regional seed source groups

1. Western sources - France and Spain have often proved to be both drought resistant and indifferent to soil type.

2. Central sources - Corsica and Italy grow well and have good form, but all need high humidity and grow poorly on limestone soils.

3. Eastern sources - The Balkan and Crimean regions appear to grow well on poorer limestone soils.

In provenance tests in the North Central United States, trees which grown from seed sources in the eastern half of the species' natural range were the most winter hardy and fastest growing, but those from Western Europe were more susceptible to frost damage (Wheeler, et al., 1976). In a provenance test a disease-resistant seed source from Yugoslavia had the fastest growing trees (Van Haverbeke, 1986).

2.1.2. Seed germination

"Germination is defined as the emergence and development from the seed embryo of those essential structures which are indicative of the seed's capacity to produce a normal plant under favorable conditions" (Willan, 1985).

Germination consists of absorption of water mostly by imbibition, causing a swelling of the seed and ultimately splitting of the seed coat, enzymatic activity and increased respiration and assimilation rates which signal the use of stored food and translocation to growing regions and cell enlargement and divisions resulting in emergence of radicle and plumule (Baskin & Baskin, 2014).

In most of seeds the radicle of the embryo is close to the micropyle, where absorption of water is easier and quicker than through the seed coat. When the radicle swells, it exerts pressure on the seed coat which commonly splits first at this point to free the radicle. This gives the rise to the primary root which grows down into the soil or substrate and soon produces lateral roots. Subsequent stages depend on whether the species exhibits epigeal germination (the hypocotyl elongates and the cotyledons are lifted above ground such as *Pinus* species) or hypogeal germination (the hypocotyl is undeveloped and the cotyledons remain on or in the ground like *Quercus* species). In epigeal germination cotyledons may also perform a valuable photosynthetic function during early growth of the seedling (Willan, 1985).

2.1.3. Seed dormancy

Seeds are the important reproductive structures of plants, which need to survival, and have evolved many mechanisms to ensure their survival. Seed dormancy is one such mechanism (Bonner, 2008).

"Dormancy is defined as a physiological state in which a seed disposed to germinate does not, even in the presence of favorable environmental conditions" (Bonner, 2008). Mainly comes two forms of dormancy: seed coat (external) dormancy and embryo (internal) dormancy. In seed coat dormancy, a hard seed coat does not allow water to penetrate. Locust and many other ornamental trees and shrubs show evidence of this type of dormancy. Scarification is used to break or soften the seed coat. Naturally, scarification is accomplished by means such as the heat of a forest fire, digestion of the seed by animals (bird or mammal) or partial break down of the seed coat by fungi or insects. This can be done mechanically by nicking the seed coat with a file, or chemically by softening the seed coat with sulfuric acid. Embryo dormancy is common in ornamental plants, and some fruit plants such as an apple. These seeds must go through a chilling period before germinating. To break this type of dormancy, stratification is used. This process involves storing seeds in a moist medium at temperatures between 0° and 10 °C. According to the species the length of time required varies. Even when environmental requirements for seed germination are met and dormancy is broken, other factors also affect germination: Age affects seed viability- younger seed is more viable than older seed. If older seed does germinate, the seedlings are less vigorous and grow more slowly than seedlings from young seeds (Bonner, 2008).

2.1.4. Seed treatments

Dormancy is a big advantage when seeds storing but it is a disadvantage when prompt germination in desired. Most of seed treatments done for break the seed dormancy. Methods used to overcome seed coat dormancy are communally known as scarification. Cold water soak, hot water soak, hot wire or burning, acid treatment mostly with sulfuric acid and mechanical treatments are some of the commonly used scarification methods. Stratification (chilling) is one of the most common methods used to overcome the internal seed dormancy.

Incubation and stratification is one of the best methods to beat the embryo dormancy or morphological dormancy (Bonner, 2008).

2.1.5. Seed testing

Seed testing is done to assess seed lot attributes to determine overall quality and value for seedling production and storage. Seed testing standards provide set procedures for facilities to conduct tests in a uniform manner to ensure comparable results that are within acceptable ranges. The seed testing standards described in this work are closely aligned with International Seed Testing Association (ISTA). International Rules for Seed Testing or Methods and Procedures for Testing Tree Seeds in Canada. (Annon, 2009)

2.1.5.1. Germination tests

The objective of the germination test is to determine the maximum germination potential of a seed lot. The percentage of seeds that develop into normal seedlings under specified conditions in a specified period of time consider as a germination percentage. Those results can be used to compare the quality of different lots and also estimate the field planting value of a seed lot (Minister of State Hayes, Irish, 2015). Germination test can be also used to evaluate the application of external chemicals such as phytohormone to test their effects on seed germination.

2.1.5.1.1. Requirements for germination tests

Seeds require some precise conditions for normal germination. The most important germination requirements are substrata, moisture, temperature and light.

The seeds can germinate and the seedlings grow well in the substrate and it serves as a moisture reservoir and provides a surface or medium. The most commonly used substrates are papers such as filter paper or blotter papers, cotton cloth, sand and soil with the pH range (Covington, et al., 1985) of 6.0 to 7.5 and free from any other seeds and the pathogens (Aswathaiah, et al.,).

During germination test standard germination containers must be used for adequate and uniform spacing. Suitable paper substrate must be selected with an open and porous nature and the capacity to hold sufficient amount of water for the duration of the test period.

Germination cabinets/chambers are also important equipment that can control temperature and light (Annon, 2009).

"Each test must consist of four hundred seeds which are drawn from the pure faction of seed and then randomly divided into four replicates of 100 seeds. Seeds for each replicate are placed on moist substrate in a germination container" (Annon, 2009).

Germinated seedlings must be assessed according to vigour classes (Figure 1). Except *Populus* spp. and *Pinus albicaulis*, all other species of germinated seedlings are assessed at weekly intervals during the specified germination test period. The number of germinated seedlings without abnormalities that reach vigour classes 1 and 2 are counted, recorded, and removed from the containers during the weekly assessments. On the final assessment day all remaining germinating seeds in vigour classes 1–7 are counted and recorded. Not only that, the number of abnormal germinating seeds, i.e. albinos, dwarfed hypocotyls, multiple stems and stunted roots must be counted and recorded. The number of un-germinated seeds must be counted and recorded on the last day of assessments (Annon, 2009).

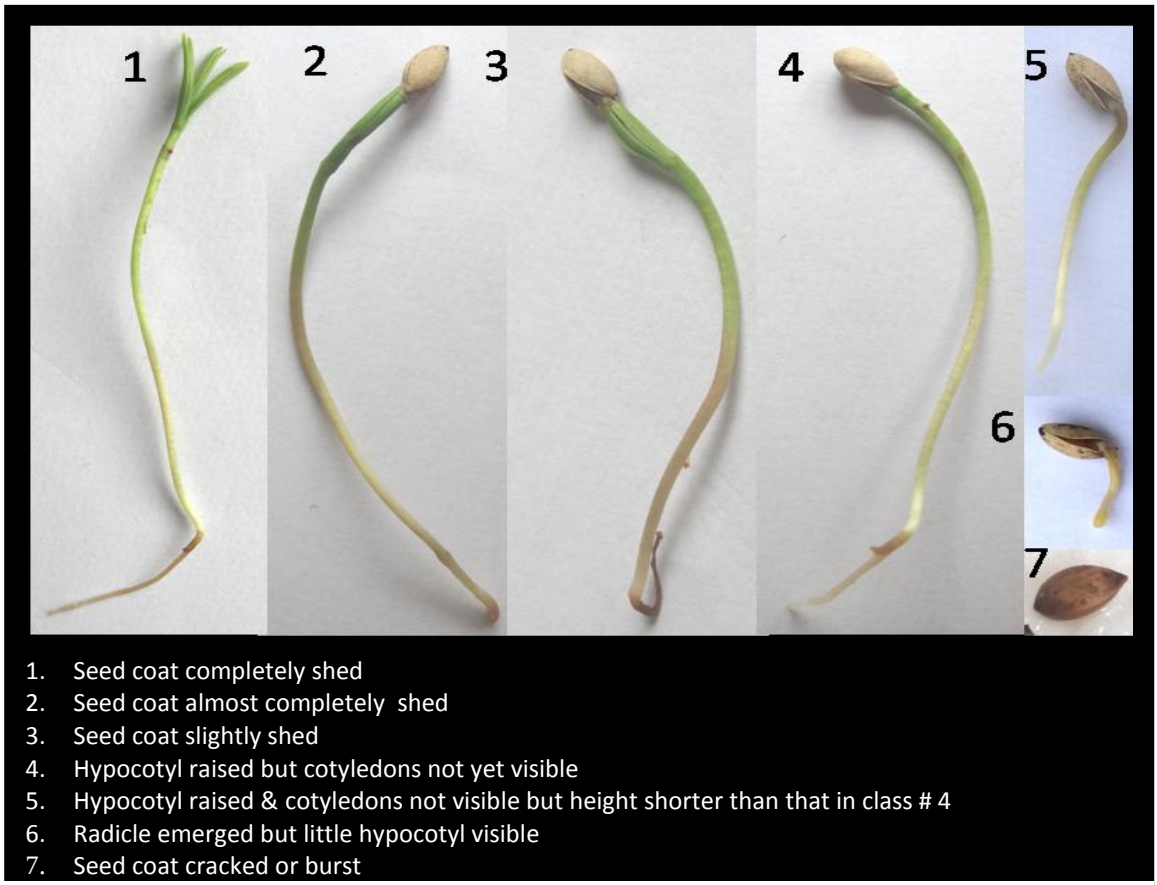


Figure 1: Laboratory germination seed vigour classes
Source: Author generated

2.2. Plant hormones

2.2.1. Overview

Plant hormones are chemicals produced within the plant that regulate the growth processes of plants. Plant hormones are also known as phytohormones or plant growth substances. Those occur in extremely low concentrations and are effective in parts per million or parts per billion in a plant. Different hormones affect different plant processes such as formation of stems, leaves, flowers, the development and ripening of fruits and the senescence of leaves, and fruits. Plant hormones also affect seed growth, time of flowering, the sex of flowers, and the shape of plants, plant longevity, and even plant death (Bewley & Black, 1995). Therefore plants need hormones at very specific times, during plant growth and at specific locations.

In general, there are five major classes of plant hormones; some of them are made up of many different chemicals that can vary in structure from one plant to another. The chemicals are grouped together into one of these classes based on their effects on plant physiology and on their structural similarities. Other plant hormones and growth regulators are not easily grouped into these classes; they exist naturally or are synthesized by humans or other organisms, including chemicals that inhibit or induce plant growth or interrupt the physiological processes within plants.

Auxins, Gibberellins (GA), Cytokinins, Ethylene and Abscisic acid (ABA) are the major groups of plant hormones. Each group of plant hormones has positive (stimulating) as well as negative (inhibitory) functions (Bewley & Black, 1995).

Auxins are produced in rapidly growing terminal or apical buds and they suppress the growth of lateral buds, which is call apical dormancy. They also have effects on tropism (control the direction of plant growth by cell elongation), fruit retention on a tree and fruit drop and they stimulate root growth. Specific protein synthesis is regulated by auxins within the seeds (Bonner, 2008).

Gibberellins are produced in the root growing tips and they stimulate shoot growth. These plant hormones affect the rate of cell divisions and flowering, they increase the size of leaves and fruits, influence seed and bud dormancy and induct growth at lower temperatures.

During seed germination gibberellins initiate mobilization of storage materials within the seed and break ABA induced dormancy (Steber & Mc Court, 2000).

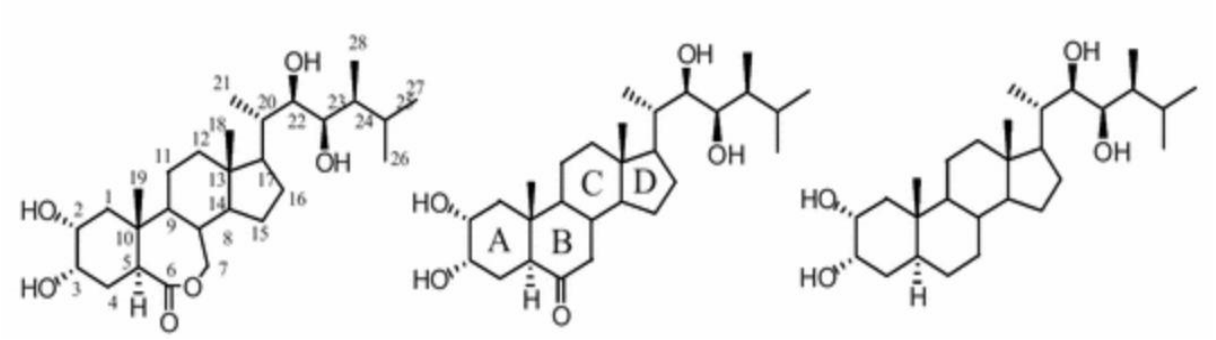
Promotion of cell division takes place by cytokinins and it influences cell differentiation and aging of leaves too (Bonner, 2008).

Ethylene production in afresh germinated seedlings is higher than can escape the plant, which leads to elevated amounts of ethylene, also inhibiting leaf expansion. Production of ethylene greatly increases, preventing cell elongation and causing the stem to swell also cell growth and cell shape. It affects stem diameter and height and play major task in fruit-ripening. Ethylene can stimulate germination as well as reverse ABA induced dormancy (Steber & Mc Court, 2000).

ABA is considered as the stress hormone and it inhibits the effects of other hormones to reduce growth during times of plant under stress. During fruit maturation ABA accumulates within seeds and preventing seed germination within the fruit and it establishes dormancy during embryo maturation (Steber & Mc Court, 2000) or seed germination before winter in temperate area plants. ABA levels decrease just before the seed germinates and during seed germination and early growth of the seedling (Bewley, 1997).

2.2.2. Brassinosteroids (BRs)

Brassinosteroids are a group of naturally occurring plant growth regulators, which exhibit structural similarities to animal steroid hormones (Singh & Shono, 2005). BRs are widespread in nature and are found in algae, gymnosperms and angiosperm plants and are present in nearly every part of the plant with the highest concentrations especially in pollen and immature seeds than in other parts of the plant (Khripach, et al., 1999; Clouse & Sasse, 1998; Khripach, et al., 2003). Chemically BRs are a class of polyhydroxysteroids, (Figure 2). BRs have been recognized as a sixth class of plant hormones which stimulate cell elongation and division, gravitropism, resistance to stress and xylem differentiation and they inhibit leaf abscission (Rao, et al., 2002; Hardtke, et al., 2007; Bajguz & Shamsul, 2009). Brassinolide was the first identified BR (Mitchell, et al., 1970) and was isolated in a crystalline form from organic extracts of rapeseed (*Brassica napus* L.) pollen in 1979. (Grove, et al., 1979).



Brassinolide (Bl) - BR-lactone type | Castasterone (Bk) - BR-ketone type | 6-Deoxocastasterone (Bd) - BR-deoxo type

Figure 2: Three type of cycle brassinosteroide in BR's series
Source: Khripach et al., 2003

The presence of BRs has been confirmed in almost every part of plants from roots to shoot, such as pollen grains, flower buds, fruits, seeds, vascular cambium, leaves, shoots and root tips. These compounds occur in free form and conjugated to sugars and fatty acids. BRs are required for normal development of plants and it is one of the major phytohormone. BRs play a critical role in a range of developmental processes such as floral initiation and development of flowers and fruits, stem and root growth on higher plants (Bajguz & Shamsul, 2009).

2.2.2.1. Brassinosteroids activities on plants

BRs have a positive as well as negative relationship with many phytohormones within the plants. They increase ethylene production in plants, have an additive effect with gibberellins; also reduce ABA response in plants over expressing. ABA is a known antagonist of BRs signaling too. BRs show synergistic effect with auxin on stem segment elongation (Bajguz & Shamsul, 2009). Co-application of BRs and GA, or BRs and auxin, resulted in a synergistic increase in hypocotyl elongation in *Arabidopsis thaliana* seedlings and ABA and BRs in cell elongation has been shown in *Arabidopsis thaliana* (Friedrichsen, et al., 2002; Tanaka, et al., 2003).

The effects of successive brassinosteroid analogue (BR) applications were evaluated in the first year production of commercial yellow passion fruit (*Passiflora edulis* f. flavicarpa) orchards. The treatments were applied shortly after the first flowers appeared, two, three, four and five consecutive weeks after the appearance of the first flowers. The fruits were collected for seven consecutive weeks and fruit mass, length and diameter, soluble solid contents; pulp

mass and peel thickness were evaluated under the laboratory condition. In this study BRs treatment resulted in a 65% increase in the estimated yield of the passion fruit plants. Their results showed that BRs sprayed during a period of reproductive development can increase the number of fruits per plant (Gomes, et al., 2006).

Treatment of plants with BRs at the proper stage of their development can result in an increase of crop yield and in some cases, in an increase of its quality. These effects can be achieved by applying BRs at doses of 20-50 mg/ha and that is much less than those for the usual plant growth stimulators. For practical application the ability of BRs to increase the resistance of plant to unfavorable factors of the environment, such as extreme temperatures, drought, salinity, or pesticides, is important also (Khripach, et al., 1999).

2.2.2.2. Role of brassinosteroids on plant under stress conditions

Plants often encounter different kinds of stresses. These can be abiotic, i.e., arising from excess or shortage of physical or chemical factors, such as water logging, drought, high or low temperatures, excessive soil salinity, heavy metals, pesticides, air pollutants and other chemicals, deficiency or excess of mineral nutrients in soil, too much light and excessive shade; or biotic, i.e., imposed by other organisms like bacteria, fungi, viruses, and insects. Those stresses which plants could meet, lead to growth retardation, decrease in biomass and low production of fruit and seeds (Hopkins & Hüner, 2009). A general consequence of nearly all abiotic and biotic stresses is that they result, at particular stage of stress exposure, in an increased production of reactive oxygen compounds (ROC) such as hydrogen peroxide, superoxide radical, hydroxyl radical and alkoxyl radical in various plant species. Although under normal growth conditions, the production of ROC in cells is low, many stresses that interrupt the cellular homeostasis of cells enhance the production of ROC. When there is a serious imbalance in any cell compartment between the production of ROC and antioxidative defence, oxidative stress occurs. That leads to cell damage. The resistance of plants to oxidative stress depends on the overall balance between the production of ROC and antioxidant potential of cell (Mittler, 2002; Arora, et al., 2002). A number of phytohormones are implicated in modulating the plant responses to oxidative stress such as auxin, ABA, ethylene, salicylic acid, and brassinosteroids (Cao, et al., 2005).

2.2.2.2.1. Osmotic stresses

Salinity, drought and freeze-induced dehydration compose direct osmotic stresses; while chilling and hypoxia can indirectly cause osmotic stress via effects on water uptake and loss in plant. By having a number of physiological and developmental changes, plants respond to dehydration and low temperature conditions. They have evolved a high capacity to synthesize and accumulate of non-toxic solutes (osmoprotectants), for example proline, glycine betaine, mannitol, and trehalose. Stress conditions like drought and high salinity cause plants to produce high levels of ABA; external application of ABA also induces a number of genes that respond to dehydration and cold stress. (Bajguz & Shamsul, 2009) Under osmotic stress conditions, BRs enhanced the activity of catalase and reduced the activities of peroxidase and ascorbic acid oxidase on the sorghum (*Sorghum vulgare*) seed germination and seedling growth (Vardhini & Rao, 2003). BRs application also resulted in enhancement of seedling growth, which was evident in terms of seedling length, seedling fresh and dry weights of all the three varieties that CSH-14 and ICSV-745 (susceptible to water stress) and M-35-1 (resistant to water stress) of sorghum (*Sorghum vulgare*) viz. under osmotic stress (Vardhini & Rao, 2003). Under the drought stress in sugar-beet plants, similar kind of results have been discuss, in which a reduction of taproot weight was correlated to stress severity. Treatment with BRs fully compensated for the reduction in biomass caused by mild drought stress. Alternatively, increases in biomass was correlated with increases in acid invertase activity in young leaves, and probably provided more assimilates to the plant due to their larger sizes (Bajguz & Shamsul, 2009).

The effect of BRs on changes in root nodulation of *Phaseolus vulgaris* L. and contents of endogenous ABA and cytokinin *trans*-zeatin riboside (ZR) and nitrogenase activity has been reported (Upreti & Murti, 2004). BRs in the unstressed plants increased root nodulation, ZR content and nitrogenase activity and also ameliorated their stress induced decline in the nodulated roots. However, ABA contents in the nodules of control or stressed plants were not altered by BRs treatment. Water stress induced decline in root nodulation is associated with increase in ABA and decline in cytokinins contents in the nodulated roots. BRs have the potential to improve root nodulation and pod yield in the irrigated and water stressed plants; an effect that could be mediated through an influence on cytokinin content in the nodulated roots of *Phaseolus vulgaris* L.

2.2.2.2.2. Thermal stress

BRs promoted the cell elongation of rice (*Oryza sativa* L.) seedlings at a low temperature (15°C). Alternatively, the effect of indole-3-acetic acid (IAA) on the cell elongation was noticeably lowered, while the combination of BRs and IAA synergistically promoted the cell elongation. Treatment with BRs also enhanced the seeds germination rate and the growth after direct sowing in submerged paddy pots in a greenhouse or phytotron at low temperature. Those results suggest that BRs promotes cell elongation in young rice seedlings under low-temperature stress, and that BRs may promote germination and the early growth of rice seedlings at a low temperature in direct sowing in the inundated paddy field and in the rice nursery (Fujii & Saka, 2001). The similar capability of BRs to improve seed germination and seedling growth of maize (*Zea mays*) (He, et al., 1991; Singh, et al., 2012) and cucumber (*Cucumis sativus*) (Khripach, et al., 1999) under chilling stress has been reported.

Extreme temperatures increased stress symptoms in plant, i.e. necrotic areas on the leaves of bananas. However, the effects of thermal stress were significantly reduced in plants which were treated with a trihydroxylated spirotane analogue of BRs. Cool temperature affected leaf emergence with a significant reduction in their number, but application of BR analogue had noticeable positive effect. Plant height was also significantly reduced by both low and high temperature extremes, where the application of BR analogue was effective only in plants exposed to the warmer temperature (González-Olmedo, et al., 2005; Singh & Shono, 2005).

Application of 24-epibrassinolide (EBR) simply increased freezing tolerance of bromegrass (*Bromus inermis*) cells by 3 to 5 °C. But it distinctly enhanced cell viability following exposure from 40 to 45 °C high temperature stress. The net effect on hardening was less than that obtained with ABA. Treatment of cells with EBR increased the accumulation of a subset of ABA inducible heat stable proteins. These results confirm that EBR confers some stress tolerance to plant cells (Wilen, et al., 1995).

Another study of role of EBR in three species of *Brassica carinata*, *B. juncea* and *B. napus* on lipid peroxidation and proline under temperature stress was found. Seeds were treated 4, 14, 24, 34 and 44 °C temperature for 5 hours alone or in combination with EBR. Low and high temperatures causes stress in terms of lipid peroxidation. High temperature causes more stress as compared with low temperature stress, when temperature stress level

was rises it was observed increase in membrane damage. However the seeds treated with the EBR shows positive effect as there is decrease in the lipid peroxidation and application of EBR at all concentrations causes' significant increase in the proline content with respect to all temperature stress in all three *Brassica* species (Pradhan, et al., 2013).

Brassica napus and tomato seedlings grown in the presence of EBR were significantly more tolerant to a lethal heat treatment than control seedlings which were grown in the absence of the EBR. The basic thermotolerance of seedlings were increased by EBR. An analysis of heat shock proteins (HSPs) in *B. napus* seedlings indicated that the HSPs were not preferentially accumulate in EBR treated seedlings at the control temperature. However, after heat stress, HSP accumulation was higher in untreated seedlings which were not treated in EBR. The results of the research provide the evidence for EBR induced expression of HSPs. The higher accumulation of HSPs in EBR treated seedlings raises the possibility that HSPs contribute, to thermotolerance in EBR treated seedlings (Dhaubhadel, et al., 1999). The beneficial effect of BRs application was also observed in fruit yield, which was increased during heat stressed conditions. This increase in fruit yield was mainly due to increase in fruit number by EBR application (Singh & Shono, 2005).

In another research maize seedlings were cultivated in plastic pots on filter paper and treated with BRs in the dark or in the light. Their results showed that at BR promoted the elongation of coleoptiles and mesocotyls but retarded the growth of leaves and roots, whether in the dark or in the light. Meanwhile the mesocotyls of etiolated seedlings showed either twining or transverse geotropism when treated by higher concentrations of BRs and improved the greening of etiolated leaves at different temperatures, especially at lower temperature in light. BRs also promoted the growth recovery of maize seedlings following chilling treatment (He, et al., 1991).

2.2.2.2.3. Saline stress

Rice (*Oryza sativa* L.) seedlings which grew *in vitro* exposed to saline stress and treated with BRs showed a significant increase in the activities of catalase, superoxide dismutase and glutathione reductase and a slight increase in ascorbate peroxidase (Núñez, et al., 2003). A relationship between salt-sensitive and salt-resistant varieties of rice (*Oryza sativa* L.) treated with EBR was also reported. These BRs treatment, at least in part, improved the tolerance of salt-sensitive rice seedlings to short-term salt stress (Özdemir, et al., 2004).

Under saline stress condition the application of EBR resulted in substantial improvement in the seed germination and seedling growth of *Eucalyptus camaldulensis*. Seed germination in the presence of NaCl was enhanced by EBR, but when seedlings were grown hydroponically in same amount of salt, uptake of EBR through roots caused more damage (Sasse, et al., 1995).

Wheat germ agglutinin (WGA), a classic cereal lectin, level is increased in plants under unfavourable conditions such as fungal infection, drought and osmotic stress, salinity and hyperthermia. WGA synthesis and accumulation were controlled by involving ABA. It was demonstrated that BR's did not influence on ABA content but enhanced only the accumulation of lectin under salt stress in the roots of wheat (*Triticum aestivum* L.). It is probable that BRs could be involved in the hormonal control of the WGA level along with ABA. BRs evidently exert a protective action on wheat seedlings via a significant decrease in the salt-induced ABA and WGA accumulation in roots (Shakirova, et al., 2002).

A study was found on BRs effect on the metabolite contents (free proline, soluble proteins and RNA) of two sorghum varieties grown in two saline sites. BRs application resulted in substantial elevated levels of free proline, soluble proteins and RNA of the two varieties of sorghum plants grown in two saline sites. The study revealed that BRs was more effective in more saline site than less saline site thus indicating its ability to counteract the negative impact of saline stress (Vardhini, 2012).

Bean plants planted under sodium chloride (salt) stressed conditions in a greenhouse were observed with the BRs effects and significantly green mass were increased than the controlled and confirm the positive effect of BRs to overcome the salt stressed condition (Kohout, et al., 2002).

2.2.2.2.4. Heavy metal stress

Plant used to response many ways to heavy metal stress conditions in their environment. The *Chlorella vulgaris* cultures which treated with heavy metals and BRs show a lower bioaccumulation of heavy metals than the cultures treated with metals exclusive of BRs. A stimulatory effect of BRs after blocking the accumulation of heavy metals on the growth and development of *Chlorella vulgaris* occurs. The study shown that application of BRs to *Chlorella vulgaris* cultures reduced the impact of heavy metals stress on growth, prevented

chlorophyll, sugar and protein loss and increased phytochelatins synthesis. Concentration-dependent stimulation was observed with increasing concentration of BRs and decreasing concentration of heavy metals (Bajguz A. , 2000; Bajguz & Shamsul, 2009). The toxic effect of cadmium on photochemical pathways in rape cotyledons were eliminates by BRs and also reduced the content of heavy metal - cadmium in the seedlings of winter rape - *Brassica napus* L. cv. Talayeh (Janeczko, et al., 2005) and copper in *Brassica juncea* L.Czern & Coss- Indian mustard (Sharma & Bhardwaj, 2007; Fariduddin, et al., 2009). The accumulation of heavy metals like cadmium, copper, lead, zinc, aluminium and nickel under the influence of BRs have been studied for different agricultural plants such as barley (*Hordeum vulgare* L.), tomato (*Lycopersicon esculentum* Mill.), radish (*Raphanus sativus* L.), sugar beet (*Beta vulgaris* L.), mustard (*Brassica juncea* L.), chickpea (*Cicer arietinum* L.) and mung bean (*Vigna radiata* L. Wilczek). It was found that the application of BRs significantly reduced the metal absorption (Khripach, et al., 1999; Hayat, et al., 2007; Hasan, et al., 2008; Ali, et al., 2008).

2.2.2.2.5. Pathogenic stress

Many studies were carried out with respect to pathogenic effects and their effects on plant and BRs. In a one study; BRs induced susceptibility of the potato tuber tissues by stimulating the mycelial growth and intensity of spore formation of *Phytophthora infenstans* and by weakening the immune status of plant tissues, as evidenced by the inhibition of wound reparation. The immune inhibiting effect of BRs is a systemic and long term character. It was observed for at least 4 months after the treatment of whole potato tubers with BRs (Vasyukova, et al., 1994).

BRs induce pathogen resistance in rice plants and it can suppress rice blast- and bacterial blight-diseases (Kutschera & Wang, 2012).

A study found that, the effects of BRs against blue mould rot disease (causal organism *Penicillium expansum*) and on senescence of harvested Jujube fruits. In this research they found that BRs effectively inhibited the development of blue mould rot and enhanced the activities of defense-related enzymes. By reducing ethylene production, BRs significantly delayed fruit senescence and maintained fruit quality. They suggested that the effects of BRs on reducing decay caused by *Penicillium expansum* may be associated with induction of disease resistance in fruit and delay of senescence (Zhu, et al., 2010).

BR affect on *Fusarium* diseases of barley research were explain that the application of the epibrassinolide (EBR) to barley reduced the severity of *Fusarium* head blight caused by *Fusarium culmorum* by 86% and reduced the head blight associated loss in grain weight by 33%. Plants growth in soil amended with epiBL resulted in a 28 to 35% decline in *Fusarium* seedling blight symptoms on barley. The results of gene expression studies illustrate that chromatin remodeling, hormonal signaling, photosynthesis, and pathogenesis related genes are activated in plants as a result of growth in EBR (Ali, et al., 2013).

2.2.2.3. Application of BRs in forestry

Application of BRs in the forestry field is not as common as in the agricultural crop sector. The influence of exogenous application of BRs on survival, growth and biomass production of Scots pine (*Pinus sylvestris*) seedlings were studied. In this study seedlings were treated with a low concentration of synthetically prepared BRs and the control without any treatment. Mortality, height and root collar diameter were measured and the root-to-shoot ratio of biomass volume was determined in autumn. In the study application of BRs has shown a negative effect on height, increment in root collar diameter and survival rate of seedling compared to the control and no significant influence of BRs on biomass production was observed (Nováková, et al., 2014).

The application of 24-epibrassinolide (EBR) on seed germination and seedling growth of *Eucalyptus camaldulensis* under saline stress condition were studied and result shown that considerable improvement in the seed germination and seedling growth on it (Sasse, et al., 1995).

A study of BRs effects, on one year old *Robinia pseudoacacia* L. seedlings was cited. In this study, seedling roots were soaked in BRs solutions before planting. Survival and growth of the seedlings were determined eight months after planting. The results showed that soaking roots in BRs before planting significantly increased the survival and growth of seedlings. In the experiment, roots were soaked in BRs before planting followed by a foliar application of BRs when the seedlings leafed out. After the seedlings were established, simulate drought conditions. The results showed that BRs treated seedlings decreased the transpiration rate, stomatal conductance and malondialdehyde content of seedlings growing under water stress compared to untreated. Leaf water content, predawn water potential, soluble sugar content, free proline content, and superoxide dismutase, peroxidase and catalase activities were all

greater in water-stressed seedlings in BRs treatment when compared with control. Their results indicate that the application of BRs can ameliorate the effects of water stress and enhance drought resistance of *Robinia* seedlings and they suggested that the treatment of seedlings with BRs perhaps a useful management tool for afforestation in arid and semiarid areas (Li, et al., 2008).

A study found that used of BRs on *Pinus wallichiana* A.B. Jacks (Himalayan blue pine or Bhutan pine). Among all the Indian pines, *Pinus wallichiana* is one of the most recalcitrant species to *in vitro* propagation *via* somatic embryogenesis. Their study highlights for the first time the successful brassinolide-mediate stimulation of embryogenesis in all the tested genotypes of *Pinus wallichiana*. 24-epibrassinolide at 2.0 µM with 9.0 µM 2, 4-D enhanced the formation of embryonic tissues from mature zygotic embryos on half strength MSG basal medium and frequency of somatic embryogenesis was not similar in tested genotypes (Malabadi & Nataraja, 2007).

The germination capacity of *Ailanthus altissima* seeds were tested with different concentrations of natural brassinolide (NBR). In that research authors found that, the germination rate and germination energy of the seeds increased by 17.6% and 18.8%, and the mean germination speed (i.e., germination time) of the seeds was shortened by 1.4 d under the optimal concentration (0.4 mg/l) treatment, compared with the control. They found that, after hypocotyls of *Ailanthus altissima* were treated with NBR, the elongation of the hypocotyls increased and among different concentrations of the NBR, 0.4 mg/l NBR appeared to be the optimal concentration for the elongation of *Ailanthus altissima* hypocotyls (Kai-rong, et al., 2005).

Somatic embryogenesis (SE) is one of the most desirable technologies for the large-scale production of high value coniferous trees from advanced breeding and genetic engineering programs. Initiation in loblolly pine (*Pinus taeda* L.), is often recalcitrant for desirable genotypes and initiation percentages of loblolly pine, Douglas-fir (*Pseudotsuga* menziesii Franco), and Norway spruce (*Picea abies* L., Karst.) were improved through the use of brassinolide. In that study media, brassinolide was effective at concentrations ranging from 0.005–0.25 mM. Using control medium (no brassinolide) and brassinolide-supplemented (0.1 mM) medium, they achieved improved initiation percentages in loblolly pine, Douglas-fir, Norway spruce, and rice from 15.0% to 30.1%, 16.1% to 36.3%, 34.6% to 47.4%, and 10%, respectively. Brassinolide increased the weight of loblolly pine embryogenic tissue by 66%

and stimulated initiation in the more recalcitrant families of loblolly pine and Douglas-fir, consequently compensating somewhat for genotypic differences in initiation. Loblolly pine initiation percentages were improved through the combination of modified 1/2-P6 salts, 50 mg/l activated carbon, adjusted levels of Cu and Zn to compensate for adsorption by activated carbon, 1.5% maltose, 2% myoinositol, 500 mg/l casamino acids, 450 mg/l glutamine, 2 mg/l a-naphthaleneacetic acid, 0.63 mg/l 6-benzylaminopurine, 0.61 mg/l kinetin, 3.4 mg/l silver nitrate, 0.1 mM brassinolide, and 2 g/l Gelrite. They come across 12 open-pollinated families of loblolly pine, initiation percentages ranged from 2.5% to 50.7%, averaging 22.5%. Finally they found that, brassinolide promoted plant embryogenic tissue formation and growth at very low concentrations and was most stimulatory at a concentration of 0.1μM. Brassinolide shows much guarantee for use with conifer and non-coniferous plants to improve SE technology for commercial propagation of high-value forest tree genotypes and for improved efficiency in plant genetic engineering (Pullman, et al., 2003).

BRs stimulated the branch elongation of *in vitro*-grown shoots of *Malus prunifolia*, (apple) rootstock research were come across. In addition to that, they observed that the BR-stimulated branch elongation was paralleled by an increase in ethylene release. The positive effect on the apple shoot growth was found. They recommend that the study was potentially useful to improve micropropagation techniques for other plant species as well, especially woody species, in which branch elongation is typically a constraint for efficient micropropagation (Pereira-Netto, et al., 2006).

3. Materials and Methods

3.1. Location

The research study was conducted from January to February 2015 in Truba research laboratory under the Department of Silviculture, Faculty of Forestry and Wood Sciences of the Czech University of Life Sciences, Prague.

3.2. Methodology

For this research, the European black pine (*Pinus nigra*) was chosen and the effect of brassinosteroid-2α,3α,17β-trihydroxy-5α-androstan-6-one that was a synthetically prepared analogue of 24-epibrassinolid (Kohout, et al., 2002) on the seed germination was tested under the conditions specified further in the text. Altogether 10,000 seeds were examined under the controlled conditions. The seeds of four BRs treatments were soaked in BRs solutions of four concentrations. The concentrations of BRs solutions were categorized as High (H) = 0.4mg/l, Medium (M) = 0.04mg/l, Medium-Channa (Mc) = 0.02mg/l, and Low (L) = 0.004mg/l. Control (C) seeds were only soaked in distilled water. The duration of the soaking process lasted 24 hours in all treatments.

Each treatment included 2,000 seeds divided into 10 vessels (containers). It means that every container included 100 seed of *Pinus nigra*. Seeds of each treatment including the control were exposed to two cultivation regimes, one half of the seeds (5 containers of each treatment) were subjected to a temporal heat stress (S) and another half was kept under optimal environmental conditions (O). Both cultivation regimes were induced in the growth chamber. Altogether entire research included ten different treatment-regime combinations and each of these combinations included 1,000 seeds (Figure 3).

3.2.1. Selection of seeds

For this research seeds of *Pinus nigra* were selected with the approved level of germination percentage, purity, and the certification. Also the seeds had been checked and approved according to the Czech Republic seed standards.

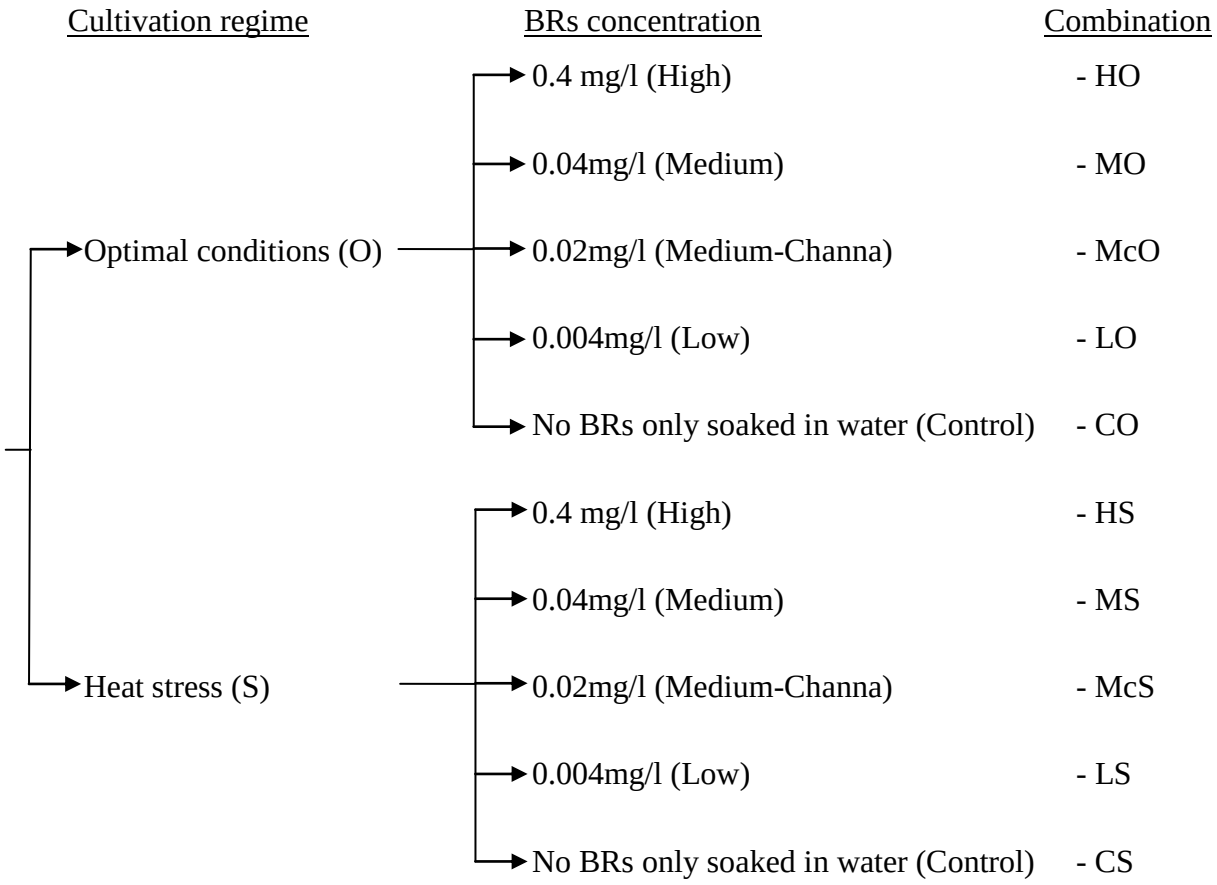


Figure 3: BRs treatment combinations with environment conditions

3.2.2. Seed preparation

Slightly more than 10,000 seeds were prepared for the study according to the weight of 1,000 seeds and they were separated into five equal lots. Seed lots were carefully washed by using distilled water and the dust and some other foreign materials which were contained in that lot were removed and clean seeds. Seeds were sterilized in Sodium hypochlorite (1% solution) and subsequently immersed in the particular soaking solutions according of the BR compound (or water in the control) for 24 hrs.

3.2.3. Preparation of germination containers and seed sowing

Germination containers were prepared for sowing of the seeds as follows: Initially the containers were cleaned and disinfected. Filter papers and other tools such as forcipes, needles, knifes etc., which were necessary for sowing the seeds, were disinfected by subjecting them into the high pressure saturated steam in oven with 121 $^{\circ}$C (249 $^{\circ}$F) for 15 to 20 minutes. Afterwards, the filter papers were cut in to container bottom size and two hard filter papers were placed in the bottom of the container and one soft filter paper was placed

above the hard filter paper in each container. Distilled water was applied to the filter papers to keep adequate amount of moisture to ensure seed germination. A label was fixed to each container to identify the affiliation to a particular treatment-regime combination. Seeds which were soaked for 24 hours in a certain concentration were sown in germination containers. In each container, 100 seeds were placed on top of three flat wetted filter papers. Seeds were placed evenly within clusters per five. The seeds in clusters did not touch each other (Figure 4). Altogether twenty seed clusters were included in each container; the clusters were arranged in four columns and five rows. This arrangement enabled easier counting. The containers were covered with plastic translucent cups protecting the seeds and keeping high moisture inside.

Figure 4: Clustered arrangement of seeds in a container

After sowing the seeds, the containers were placed in two growth chambers, which were programmed for two different treatment regimes: 1) optimal environmental conditions (O) and 2) stressed environmental conditions (S). A cycle of 24 hrs under the optimal environmental conditions (regime O) was programmed as follows: light period lasted for eight hours with the following adjustments: the temperature was kept at 30 $^{\circ}$C and the light intensity was ranging between: 11,200 lx and 13,050 lx. The dark period in the chamber was set up for sixteen hours and temperature of 20 $^{\circ}$C. A maximum humidity was kept inside the containers. The cultivation in the chamber lasted altogether 28 days.

For the stressed conditions (Regime S), a temporal heat-stress segment lasting 10 hrs was included on the sixth day of the cultivation: During the first hour the temperature increased

from 30°C to 37°C. After the following 30 minutes, during the second hour the temperature increased from 37°C to 40°C. During the third hour the temperature increased from 40°C to 42°C, during the fourth hour the temperature stayed constant and equaled 42°C (heat stress peak), during the fifth hour the temperature was already decreasing form 42°C to 40°C, during the sixth hour the temperature decreased from 40°C to 37°C and during the seventh hour from 37°C to 30°C. In between each temperature hour, 30 minutes gap was kept and altogether 10 hours light period was maintained as day time (Figure 5). The remaining part of the cultivation process was the same as in the optimal regime. The seeds were kept in the growing chamber for four weeks (28 days) from the sowing of seeds.

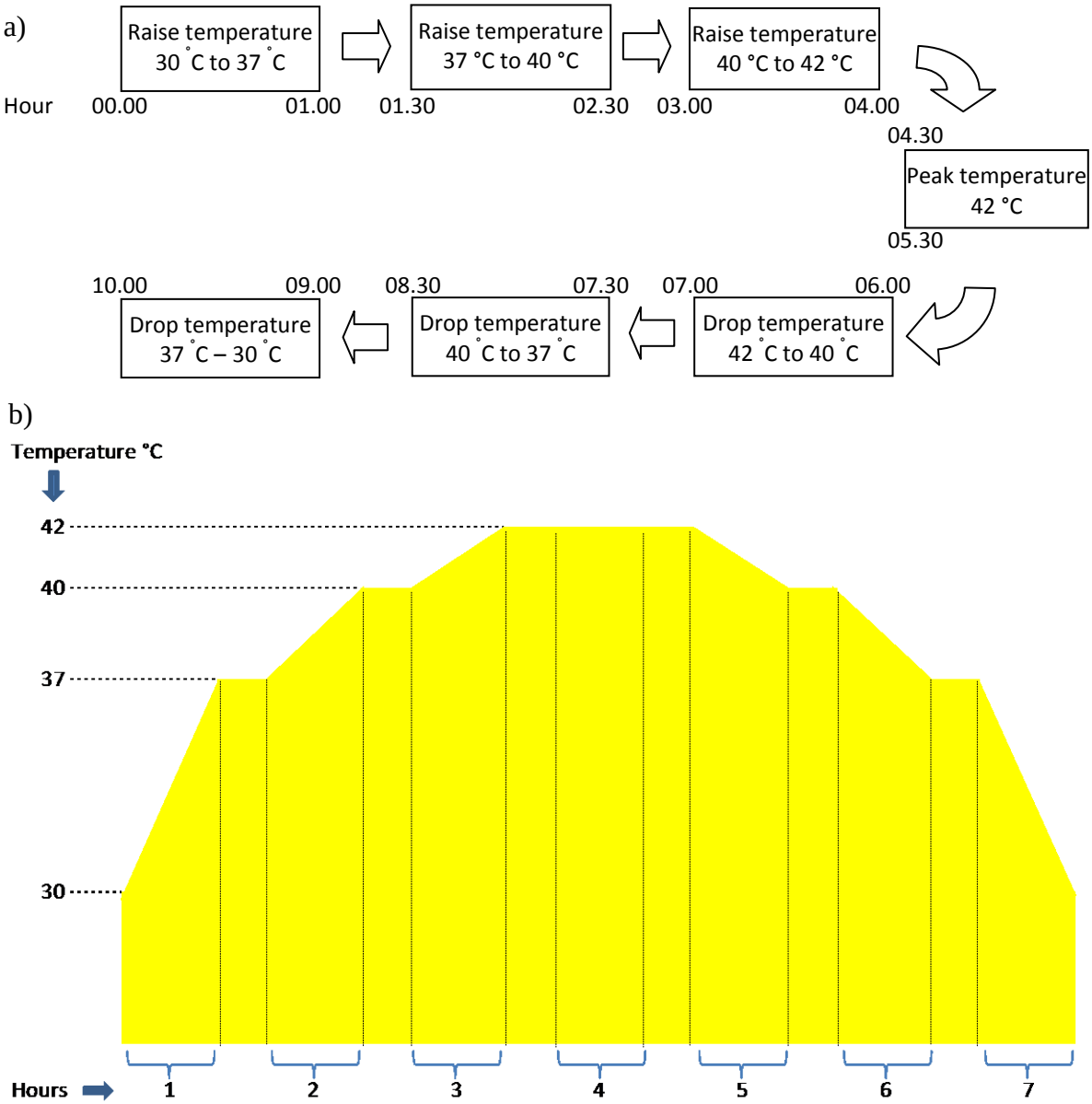


Figure 5: Temporal heat-stress segment under stressed regime.

a) Heat stress segment hourly changes
b) Temperature variation and stabilized periods

3.2.4. Evaluation of seed germination

Seed germinations were evaluated in seven-day intervals. First counting was done seven days after seed sowing and it was repeated regularly in the fourteenth, twenty-first and twenty-eighth day after seed sowing (resp. container placement to the growth chamber). Germinations were evaluated at each container according to the following categories. First of all, the rotten seeds were counted and removed from the container and after that living seeds were counted, but only the ones which did not start to germinate. After that, the seeds that already had started to germinate but had not reached full germination were counted. All the living seeds and the seeds which already started to germinate were kept in containers for further growing. Finally, completely germinated seeds were counted and they were removed from the container. According to the International Seed Testing Association (ISTA) standards, if the length of radicular is four times longer than the length of the seed, then the seedling is considered as fully germinated (Annon, 2009) (Figure 6). Length of radiculas of fully germinated seeds ware measured separately and recorded in a data sheet (Appendix 1. Model of data recorded sheet).

Figure 6: Fully germinated seed (Scale in centimeters with millimeter accuracy)

3.2.5. Analysis of data

The statistical analyses were conducted in STATISTICA12 (StatSoft Inc., Tulsa, USA) software. For analysis of twenty eighth day germination capacity of optimal and stressed regimes of seed germination, multiple comparisons of binomial distribution data were used (Agresti, et al., 2008). Kruskal-Wallis test with multiple comparisons of radicular length was used to analyze the seventh day germination energy.

4. Results

4.1. Germination capacity

Optimal regime and stressed regime data were analyzed separately and multiple comparisons were performed within the groups of treatment-regime combinations (Table 1 and 2.). Fully germinated seeds were only used for multiple comparisons.

The multiple comparisons of all combinations with references to germination capacity and BRs concentrations under optimal conditions are shown in Table 1.

Table 1: Multiple comparisons results of germination capacity of germinated seeds in the compared treatments under optimal conditions in the 28th day.

Compared pairs of treatments after multiple comparison		Test.stat value	Critical value	p-value
WO	LO	1.0952	3.8577	0.938
WO	McO	4.7337	3.8577	0.007
WO	MO	7.0933	3.8577	<0.001
WO	HO	13.2999	3.8577	<0.001
LO	McO	3.6339	3.8577	0.076
LO	MO	5.9867	3.8577	<0.001
LO	HO	12.1605	3.8577	<0.001
McO	MO	2.3402	3.8577	0.462
McO	HO	8.4332	3.8577	<0.001
MO	HO	6.0632	3.8577	<0.001

Note: WO=Control, LO=low concentration, McO=Medium-Channa concentration, MO=Medium concentration, HO= high concentration

Under optimal regime (Table 1), no significant difference in germination capacity was recorded between the control treatment (WO) and low concentration treatment (LO) (p-value = 0.938). However, the other concentration levels of BRs (McO, MO, HO), if confronted with the control, showed significant differences (0.007, <0.001 and <0.001). Comparisons of germination capacity of seeds between the control (WO) and high concentration (HO) as well as medium (MO) concentrations of BR showed very clearly significant differences and it suggests that BRs can help to increase the germination capacity of *Pinus nigra* seeds.

Comparison of low concentration (LO) and Medium-Channa (McO) concentration showed insignificant difference (p-value = 0.076). As it was in the control (WO), low concentration treatment (LO) showed significantly lower germination capacity of germinated

seeds in the day of 28 when compared with high concentration treatment (HO) and with medium concentration treatment (MO). Both of these comparisons showed significant differences (<0.001 and <0.001 respectively). Between Medium-Channa (McO) and medium concentrations (MO) there was no significant difference (p-value = 0.462) but the comparison of McO and MO showed significant difference when compared with high concentration (HO). The p-value in both cases was <0.001.

Left side of Figure 7 (blue color) shows the percentage of germinated seeds under the optimal conditions with respect to each BRs concentration.

Results of multiple comparisons of germination capacity in the compared treatments under the stressed conditions are shown in Table 2.

Table 2: Multiple comparisons results of germination capacity of germinated seeds in the compared treatments under stressed conditions in the 28th day.

Compared pairs of treatments after multiple comparison		Test.stat value	Critical value	p-value
WS	LS	3.4141	3.8577	0.112
WS	McS	6.0674	3.8577	<0.001
WS	MS	18.5008	3.8577	<0.001
WS	HS	6.7792	3.8577	<0.001
LS	McS	2.6951	3.8577	0.314
LS	MS	15.3643	3.8577	<0.001
LS	HS	3.4216	3.8577	0.110
McS	MS	12.761	3.8577	<0.001
McS	HS	0.7298	3.8577	0.986
MS	HS	12.0447	3.8577	<0.001

Note: WS=Control, LS=low concentration, McS=Medium-Channa concentration, MS=Medium concentration, HS= high concentration

According to the study results shown in Figure 7, germination capacity of seeds under the optimal conditions and stressed conditions show increasing positive trend while increasing BR concentration, except high concentration of stressed condition. Also under stressed regime, there was no significant difference between the control (WS) and low concentration treatment (LS). However, control (WS), significantly differed from all the other BR treatments. When comparing low concentration treatment (LS) with Medium-Channa (McS) treatment as well as high concentration treatment (HS) p-values were higher than 0.05, which means that between these two pairs of concentrations (LS *vs* McS and LS *vs* HS) there were no significant differences. Clear significant differences were obtained when medium

concentration treatment (MS) was compared with low concentration treatment (LS), medium-Channa (McS) treatment and high concentration treatment (HS), respectively. The low concentration treatment, medium-Channa treatment and high concentration treatment did not differ significantly from each other.

All the above groups of multiple comparisons under both optimal conditions and stressed conditions are shown in Figure 7. Same letters in the graph bellow denote statistical homogeneity of the groups.

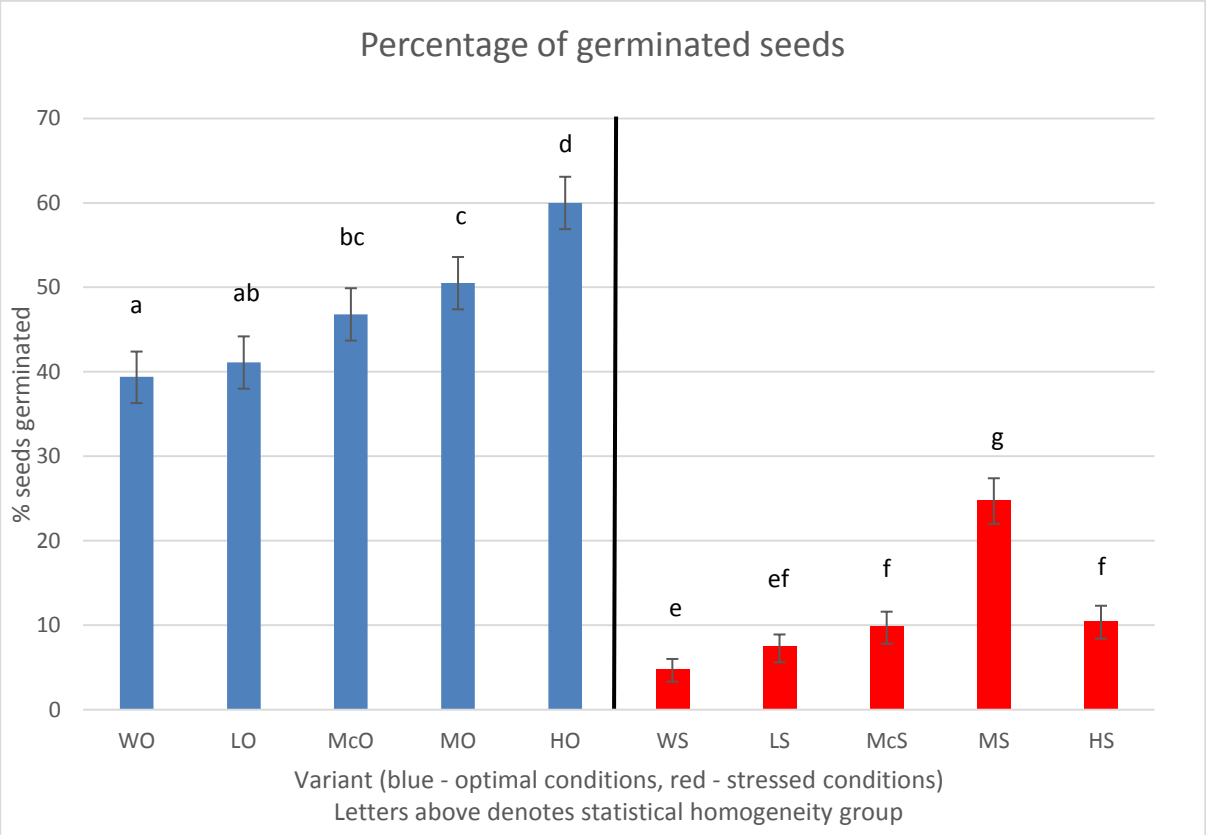


Figure 7: BR effects on seed germination under optimal condition and stressed conditions in the 28th day. Error bars depict the 95% confidence intervals.

Figure 7 shows the positive effect of BR on seed germination. Except for high concentration treatment cultivated under stressed conditions, increasing the BR concentration positively affected the germination capacity of seeds even under the stressed conditions. Confidence intervals are shown in the above graph using error bars. Under optimal conditions it fluctuates in between 3 and 3.1 and under stressed conditions it fluctuates in between 1.2 to 2.8. Above graph, error bar limits to 95%, lower and upper confidence intervals are shown in Table 3.

Table 3: Confidence intervals of lower and upper limits of treatment combinations

Treatment combination	95% lower limit	germinated percentage	95% upper limit
WO	36.4	39.4	42.5
LO	38.0	41.1	44.2
McO	43.7	46.8	49.9
MO	47.4	50.5	53.6
HO	56.9	60.0	63.1
WS	3.6	4.8	6.3
LS	5.9	7.4	9.2
McS	8.0	9.8	11.8
MS	22.2	24.8	27.6
HS	8.7	10.5	12.6

Note: WO=Control Optimal conditions, LO=Low concentration Optimal conditions, McO=Medium-Channa concentration Optimal conditions, MO=Medium concentration Optimal conditions, HO= High concentration Optimal conditions WS=Control Stressed, LS=Low concentration Stressed, McS=Medium-Channa concentration Stressed, MS=Medium concentration Stressed, HS= High concentration Stressed.

4.2. Germination energy

In this comparison also optimal regime and stressed regime data were analyzed separately. Radicular lengths of seventh day fully germinated seeds were only used for this comparisons and it was performed within the groups of variant under each treatment-regime (Figure 8 and 9).

According to the seventh day radicular length of germinated seeds under optimal regime multiple comparisons were executed (Figure 8). Under this regime, no significant difference in germination energy was recorded between the control treatment (WO), low concentration treatment (LO) and medium-channa treatment (McO). However, the other concentration levels of BR (MO, HO), if encounter with the control, low and medium-channa concentrations showed significant differences as well as high concentration and medium concentration showed the significant different among them. High concentration (HO) showed the second best germination energy performance next to the best concentration of medium (MO).

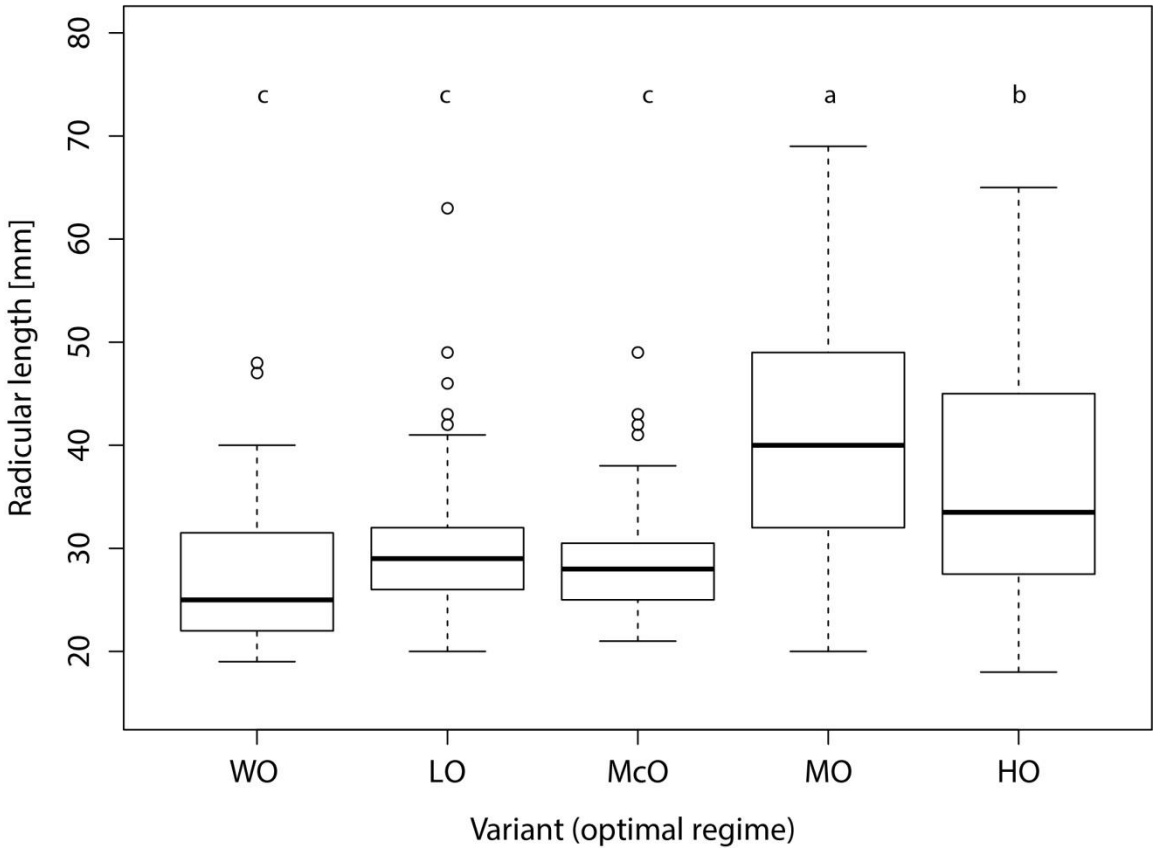


Figure 8: BR effects on germination energy in the compared variants under optimal conditions in the seventh day.
Significant differences between groups are marked with alphabetical symbols in the graph. The horizontal lines in boxplots stand for medians; the bottom and top of the box shows the 25^{th} and 75^{th} percentiles, respectively. Whiskers show either the range upper and lower values. Outliers are plotted individually.

Figure 9 shows the multiple comparisons of seventh day radicular length of germinated seeds under stressed regime. According to the box plot, no significant difference in germination energy was recorded between the control treatment (WS), low concentration treatment (LS), medium-channa treatment (McS) and high concentration (HS). However, the medium concentration (MS) of BR, showed significant difference between control treatment (WS), low concentration treatment (LS) and high concentration (HS) except medium-channa concentration. In this regime also medium concentration (MS) showed the best performance of germination energy with respect to BRs applications.

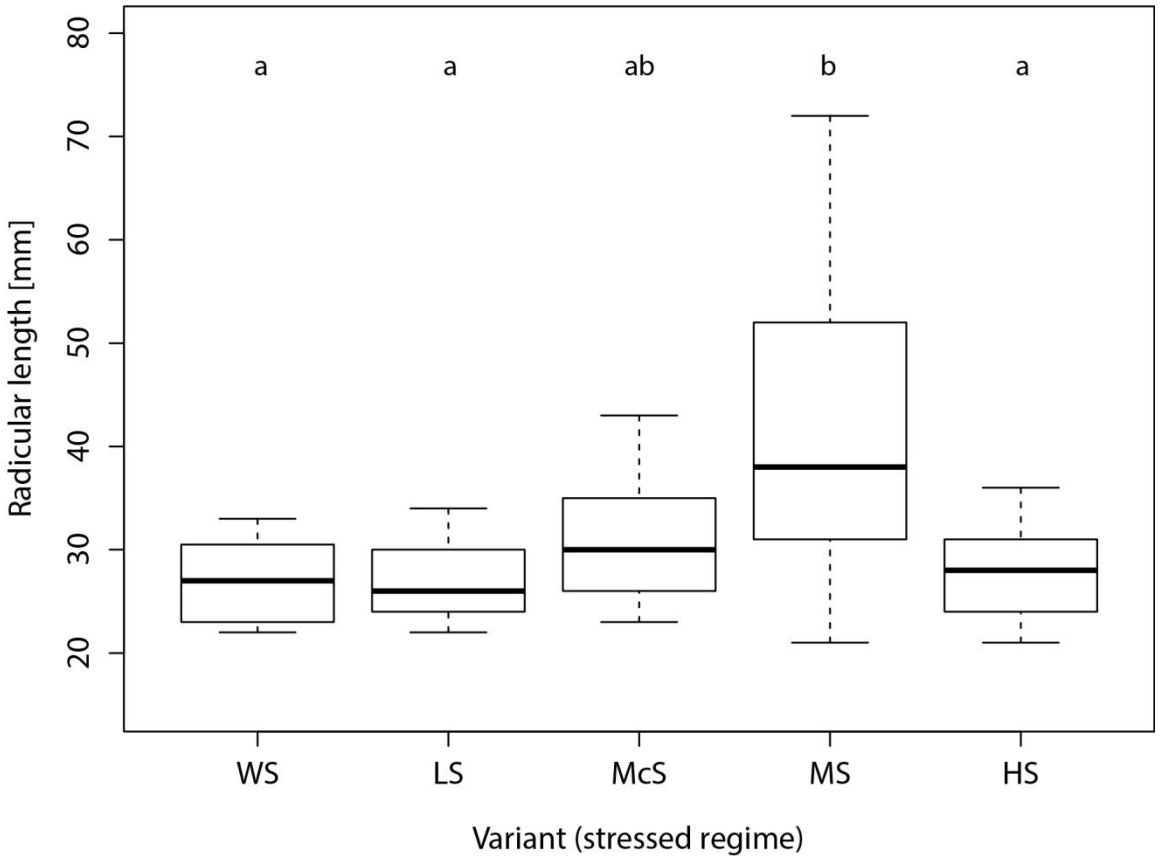


Figure 9: BR effects on germination energy in the compared variants under thermal stressed-regime in the seventh day.
Significant differences between groups are marked with alphabetical symbols in the graph. The horizontal lines in boxplots stand for medians; the bottom and top of the box shows the 25th and 75th percentiles, respectively. Whiskers show either the range upper and lower values.

According to the above results, under optimal condition and stressed condition on germination energy of *Pinus nigra* seeds show the best performance with the application of medium concentration of BRs (0.04mg/l).

5. Discussion

The aim of the study was to assess whether the exogenous application of BRs is able to improve the germination capacity and germination energy of *Pinus nigra* seeds and BRs can alleviate the stress of seed in the course of germination. As mentioned in chapter two, there was a lack of experience with BRs effects on forest trees (Nováková, et al., 2014). Vardhini & Rao, (2003) explained the BRs enhanced the activity of catalase and reduced the activities of peroxidase and ascorbic acid oxidase activities under osmotic stress conditions on the germination and seedling growth in sorghum (*Sorghum vulgare*). Núñez, et al., (2003) found that rice seedlings which grown *in vitro* exposed to saline stress and treated with BRs analogue on showed a significant increase in the activities of catalase, superoxide dismutase and glutathione reductase and a slight increase in ascorbate peroxidase.

BRs promoted the elongation of coleoptiles and mesocotyls in a maize seedling, but retarded the growth of leaves and roots. When seedling treated by higher concentrations of BRs and improved the greening of etiolated leaves at different temperatures, especially at lower temperature in light (He, et al., 1991). BRs promote cell elongation, germination and the early growth in young rice (*Oryza sativa* L.) seedlings (Fujii & Saka, 2001), maize (*Zea mays*) seedlings (Singh, et al., 2012), and cucumber (*Cucumis sativus*) seedling (Khripach, Zhabinskii, & Groot, 1999) under low-temperature stress.

On the role of brassinosteroids in thermotolerance of tomato (*Lycopersicon esculentum*) plants were studied Singh & Shono, (2005) and observed that BRs treated tomato plant survived at lethal temperature (45°C). They found that BRs treatment induced a basic thermotolerance in tomato plant. In our study we also found the similar result and BRs help to survival the *Pinus nigra* seedlings under high temperature.

The research works of Friedrichsen, et al., (2002); Tanaka, et al., (2003) reported a stimulating effect of BRs on the hypocotyl elongation and cell elongation of *Arabidopsis thaliana* seedlings. Also Tanaka, et al., (2003) mentioned that hypocotyls elongation was achieved through cell enlargement using the BRs. Somewhat similar result was observed in our study, with the increase of BRs concentration on germination of *Pinus nigra* seeds, it increase the seedlings radicular length. According to the study medium concentrations of BRs (0.04mg/l) gave the best results.

A study of BRs effects on woody plants, on Survival and growth of the *Robinia pseudoacacia* seedlings under drought conditions were studied and BRs significantly increased the survival and growth of seedlings. The results indicate that the application of BRs can improve the effects of water stress and enhance drought resistance of *Robinia* seedlings. The best results were in the 0.2 mg/l BRs treatment and authors suggested that BRs can be used in afforestation as a useful management tool (Li, et al., 2008).

Effects of BRs were studied on most recalcitrant woody species to *in vitro* propagation *via* somatic embryogenesis (SE). On *Pinus wallichiana* 24-epibrassinolide at 2.0 µM with 9.0 µM 2, 4-D enhanced the formation of embryonic tissues from mature zygotic embryos. (Malabadi & Nataraja, 2007) SE Initiation in loblolly pine (*Pinus taeda* L.), is often recalcitrant for desirable genotypes and initiation percentages of loblolly pine, Douglas-fir (*Pseudotsuga* menziesii Franco), and Norway spruce (*Picea abies* L., Karst.) were improved through the use of brassinolide under *in vitro* propagation. Brassinolide increased the weight of loblolly pine embryogenic tissue by 66%. Brassinolide promoted plant embryogenic tissue formation and growth at very low concentrations and was most stimulatory at a concentration of 0.1µM (Pullman, et al., 2003).

After application of natural brassinolide (NBR) on *Ailanthus altissima* seeds, the elongation of the hypocotyls increased among different concentrations of the NBR. 0.4 mg/l NBR appeared to be the optimal concentration for the elongation of *Ailanthus altissima* hypocotyls. The germination rate and germination energy of *Ailanthus altissima* seeds increased by 17.6% and 18.8%, and the mean germination speed of the seeds was shortened by 1.4 days under the optimal concentration (0.4 mg/l) treatment.

Application of BRs has shown a negative effect on height, increment in root collar diameter and survival rate of seedling compared to the control no significant influence of BRs on biomass production was observed on Scots pine (*Pinus sylvestris*) seedlings (Nováková, et al., 2014). However on germination capacity and germination energy on *Pinus nigra* of our study shows that, BRs can remarkably improve the germination capacity and germination energy.

6. Conclusion

The main aim of this study is to assess whether the BRs can improve the germination capacity and germination energy of *Pinus nigra* seeds. In the meantime necessitate finding out, that the BRs can alleviate the stress of seed in the course of germination. In this study found that, the germination capacity and germination energy of *Pines nigra* seeds with BRs treatment were significantly higher compared to the control. As for germination capacity, high (0.4 mg/l) concentration and medium (0.04 mg/l) concentration, respectively optimal and stressed condition showed the best result of this study. Medium (0.04 mg/l) concentration of BRs treatment with respect to germination energy on both optimal and stressed conditions was found the best performance.

Further research is needed to establish the respective merits of the BRs concentration on germination of forest seeds with respect to each species and only then can recommend the exact concentration of BRs with reference to each species.

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Appendixes

Appendix 1. Model of data sheet.

Date of established :- Name :-

Date of counted :-

Treatment	D	0	1	2	3

D = Rotten

0 = Live seeds but not germinated yet

1 = Germinate started seeds but not fully germinated

2 = Completely germinated seeds

3 = Radicular length of each germinated seed