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The Effect of external regulatory substances application on the progamic phase of male gametophyte development

Diploma Thesis

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Decleration

I declare that the Diploma Thesis “The Effect of external regulatory substances application on the progamic phase of male gametophyte development” is my own original work and all the information sources used are cited and listed as references.

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Vliv aplikace externích regulačních látek na progamickou fázi vývoje samčího gametofytu

Abstrakt

Syntetický biostimulant Atonik se používá ke zvýšení výnosu plodin, zejména u rostlin, které jsou vystaveny stresu. Mechanismy působení Atoniku na rostlinu nejsou plně prozkoumány. Jedním z možných způsobů jeho působení je zvýšení klíčivosti pylových zrn a růstu pylových láček, což vede k vyšší míře oplodnění a následně k tvorbě semen a plodů.

V této studii byl testován vliv přípravku Atonik na klíčivost pylu a růst pylových láček pomocí *in vitro* pylových kultur. Atonik byl aplikován na médium SMM-MES ve třech koncentracích 0.1%, 0.2%, 0.5% a porovnán s kontrolní variantou. Kultury byly kultivovány a hodnoceny ve třech časových intervalech; 1 hodina, 2 hodiny a 4 hodiny. Po uplynutí každého časového intervalu byla měřena míra klíčení a délka láček pylových zrn.

Výsledky po statistické analýze neprokázaly významný účinek 0.1% a 0.2% Atoniku na rychlost klíčení pylu a růst pylových láček za normálních podmínek nebo pod chladovým stresem. 0.5% Atonik vykazoval inhibiční účinek na klíčení pylu a prodloužení pylových láček s inhibičním účinkem výraznějším za chladnějších teplot.

Předpoklady uvedené v hypotéze byly vyvráceny. Získané výsledky se týkají *in vitro* kultivace pylu tabáku. Doporučuje se další studie účinků přípravku Atonik způsobem *in vivo*.

Klíčová slova: *Nicotiana tabacum*, klíčení pylu, růst pylových láček, *in vitro*, Atonik.

The Effect of external regulatory substances application on the progamic phase of male gametophyte development

Abstract

Synthetic biostimulant Atonik is used to increase crop yield, especially in plants undergoing stress. The mechanisms in which it functions are not fully explored. An increase in pollen grain germination and tube growth leading to higher rate of fertilization and in turn seed and fruit formation is one possible mode of action.

In this study the effect of Atonik on pollen germination and tube growth was tested using *in vitro* pollen cultures. Atonik was applied to SMM-MES medium in three concentrations of 0.1%, 0.2%, 0.5% and contrasted with a control variant. The cultures were cultivated for three time periods of 1 hour, 2 hours and 4 hours. After each time duration the germination rate and tube lengths of pollen grains was measured.

The results after statistical analysis showed no significant effect of 0.1% and 0.2% Atonik on both pollen germination rate and pollen tube growth under normal or temperature stress conditions. 0.5% Atonik showed inhibitory effect on the pollen germination rate and pollen tube elongation with the inhibitory effect more prominent under colder temperatures.

The assumptions stated in the hypothesis were refuted. The results obtained apply to *in vitro* pollen cultivation of tobacco pollen. Further study on *in vivo* effects of Atonik application is recommended.

Keywords: *Nicotiana tabacum*, pollen germination, pollen tube growth, *in vitro*, Atonik

Contents

1 Introduction

Biostimulants are a group of various substances, compounds and even microorganisms applied to plants to enhance their growth. The global market for them is estimated to be 2200 million dollars in 2018, of which Europe is the largest market. Opinions in the scientific community consider them to lack in peer-reviewed evaluation. However, there has been a growing body of research supporting their use to increase agricultural output (Calvo et al. 2014). Atonik is such a biostimulant and is essentially a compound of aromatic nitrophenols. This product has been used worldwide for many years and has been successfully used to increase crop yields and mitigate crop loss in stressed plants where its positive effects are most prominent. All the phases of plant development and growth are affected by Atonik, particularly generative. Positive effects have also been reported in seed germination and growth. Its exact mode of mechanism of action however have not been covered thoroughly in literature (Przybysz et al. 2014).

The progamic phase begins after pollen grains land on the stigma surface where it is activated by rehydration, swelling the grains and building osmotic pressure. This leads to germination of the pollen grains producing a pollen tube at the intine sites weakened by enzyme activities. This tube grows into the pistil tissues (Hafidh et al. 2016). The main function of the pollen tube elongation is the delivery of two male gamets into the embryo sac for double fertilization. In this event one gamet fuses with an egg nucleus while the other fuses with a pair of polar nuclei forming a zygote and endosperm creating seeds, the main product in agricultural production (Wang 2010).

Germination and tube growth of pollen is not dependent on the female tissue in many species including tobacco therefore can be easily achieved via *in vitro* cultivation. Cultivation media for pollen cultures are diverse in their composition, varying among species, in some it can even be done in plain water. However, in spite of high optimization the pollen cultured *in vitro* never reaches the parameters of pollen grown *in vivo* (Graaf et al. 2001).

2 Aim of thesis

The aim of this study was to evaluate the effect of Atonik (1 g/L sodium 5-nitroguaiacolate, 2 g/L sodium 2-nitrophenolate and 3 g/L sodium 4-nitrophenolate) biostimulant on the progamic phase of the male gametophyte of tobacco (*Nicotiana tabacum* cv. Samsun) *in vitro*.

Scientific hypothesis:

- Atonik at 0.1%, 0.2% and 0.5% concentrations in the cultivation medium has a positive effect on pollen germination of *Nicotiana tabacum*.

- Atonik at 0.1%, 0.2% and 0.5% concentrations in the cultivation medium has a positive effect on pollen tube elongation of *Nicotiana tabacum*.

3 Literature overview

3.1 Angiosperms and their reproduction

The defining characteristic of angiosperms is their flowers. They are typically hermaphroditic and contain both reproductive and sterile organs (Kramer et al. 1998). The flowers form cells that are undifferentiated, these cells grow from the shoot apical meristems, flanks. Cells of these floral primordia first divide and then they differentiate into a suitable number of floral organs, in the proper places. It is imperative for each cell during this development process to be able to determine its relative position to others, so it can differentiate accordingly (Bowman et al. 1989).

The floral sterile and reproductive organs are generally arranged into whorls, with a particular organ type on the axis of a fixed floral meristem, emerging from a sole node. *Arabidopsis thaliana* which is often used as a model species display a higher floral arrangement typical of eudicots. The sterile sepals and petals are housed respectively in the first and second whorl of the flower, while the pollen producing male reproductive structures stamens is contained in a third whorl. The carpels, the female reproductive structures which contain the ovules emerge in a forth whorl. There are many variations of floral arrangement, structure and organ number within angiosperms (Kramer et al. 1998).

The reproductive life cycle in plants (Figure 1) alternates between two generation, one is a gamete-producing multicellular haploid organism, the gametophyte, and the other is a spore-producing multicellular diploid organism, the sporophyte. Different from other plants, the gametophytes of angiosperms rely significantly on the sporophyte tissues for their development (Mascarenhas 1989). The male gametophyte development is within the anther and consists of two sperm cells enveloped within a vegetative cell. The female gametophyte development is within the ovule and comprises of one central cell three antipodal cells, one egg cell and two synergid cells (Steffen et al. 2007).

Double fertilization is ubiquitous within flowering plants and a requisite for sexual reproduction. It involves a series of complex interactions between what is essentially three plants female gametophyte, male gametophyte and sporophyte. It culminates in a fusion of nuclei and sexual cells, forming an embryo and endosperm (Kovaleva et al. 2007).

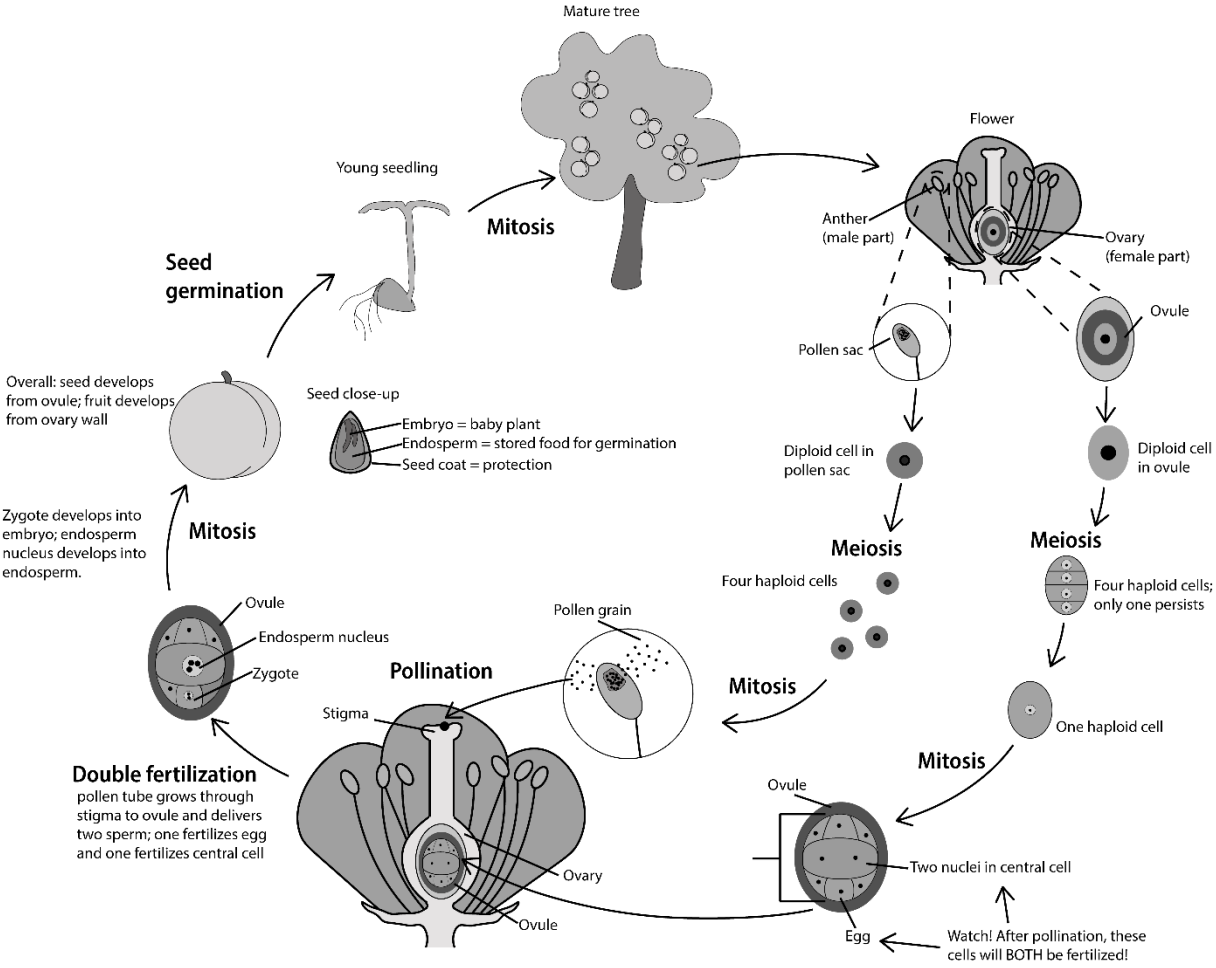

Figure 1: Life cycle of angiosperms.

Source: (Greenwood 2018)

3.2 Microsporangium

Stamen is the male reproductive organ of angiosperms. The anther houses microsporangia (pollen sacs) which is the site of pollen maturation. The filament in most species is used to transmit nutrients and water to the anther, it also provides vascular links. (Scott et al. 2004). A connective intervening is what links the anther to the filament. This intervening can be sorted into tree types. Basifixed where it differs from the tip of the

filament, dorsifixed where its inside of the broader part of the anther and versatile where the anther is able to freely turn (Eames 1961). In primitive angiosperms, there is a lack of distinction between the filament and anther (Bhandari 1984). In such cases the stamen is highly reduced such as in *Degeneria* which is regarded as the most primitive angiosperm plant (Ernes 1961). Typically, the anther of angiosperms houses four microsporangia. In some cases, it houses two microsporangia however tetrasporangiate is widely the most common and is found in more than 190 families (Bhandari 1984).

A collection of distinctive cell layers make up the anther. These layers are initiated in the anther primordium by periclinal divisions happening in the hypodermal cells. With this they are able to make archesporial cells, which settle in each of the corners of the anther primordium. Afterwards, a series of mitotic divisions in the archesporial cells undergo forming each of the unique anther cell layers. The two first layers to form are the inner primary sporogenous cells and the outer primary parietal cells. Different fates await these two layers. The primary sporogenous cells go on to form pollen mother cells by undergoing a sequence of divisions. On the other hand, the outer primary parietal cells, by also undergoing divisions morph into sporophytic cell layers forming the anther wall. This morphing process happens by the primary parietal cells dividing and forming endothecial cells and secondary parietal cells these then continue to divide forming the tapetum and the middle layer. The final resulting structure of this is a sequence of cell layers enclosing the gametophytes (Wilson and Zhang 2009).

The most common anther wall of angiosperms is made of epidermis, endothecium, middle layers and tapetum. Primitive angiosperms have a massive wall thanks to the middle layers such as *Degeneriaceae* and *Ranunculaceae*. In more advanced families the middle layers consist of only one middle layer making for a thin anthem wall (Bhandari 1984). The number of middle layers depends on if both the inner and outer primary parietal layers or only one of them join in the formation. These middle layers compress and can even be completely destroyed before the microsporangium has fully matured (Swamy and Krishnamurthy 1980).

3.2.1 Tapetum

The tapetum plays a central role in pollen wall formation, it provides it with nutrition and materials for its development (Scott et al. 2004). Its envelopment of the sporogenous tissue means that all the materials must pass through (Rudramuniyappa and Annigeri 1985). It ensures meiosis and the normal development of microspores and pollen grains. The metabolic potential of the tapetum is a lot greater than that of other cell layers and it is capable of utilizing their metabolic products. Studies have shown it is a special sporophyte structure and factors which can disturb its interaction with the gametophyte lead to male sterility (Dobrovol'skaya et al. 2009). It synthesizes enzymes such as callase, forms orbicules and viscin threads. It also contributes an exine coating which is rich with lipids in many plant species. In *Lilium* the tapetum contributes a blend of carotenins, lipids and flavonols onto the surface of the exine coating (Scott et al. 2004). This special sticky and heterogenous material of lipids, polysaccharides, flavonoids, proteins and carotenoids is called pollenkitt. This material keeps pollen grains together when they are transported, protects the pollen from ultraviolet light, exocellular enzymes, water loss, hydrolysis and even is able to maintain the sporophytic proteins inside the exine cavities (Halbritter et al. 2018).

The tapetum is considered to be the innermost anther wall layer. It reaches its peak development at the tetrad stage in microsporogenesis. In most cases it is comprised of a lone cell layer with a dense presence of cytoplasm and has a prominent nucleus (Chapman 1987).

There are typically two types of tapetum recognized in angiosperms, the glandular type and the ameboid type. In glandular tapetum the substance it secretes reach the microspores by locular fluid while the amoeboid tapetum cytoplasm adheres very close to the microspores. However, the characters of these two types may become blurred especially in later degeneration phases which result in a distinction between the two types being extremely difficult (Pacini et al. 1985). Four other subtypes can be distinguished depending on the if the periplasmodium is established, when meiosis in pollen mother cell is underway (triglochin), it was formed at the tetrad phase (sparganium and butomus) or the separation of the microspores have passed (sagittaria) (Bhandari 1984).

Break down of the tapetum begins in the later phases of pollen development, before mitosis I begins and after the microspores are released from the tetrad. This breakdown process is well regulated and has the indications of programmed cell deaths and the shrinkage of the cytoplasm after its disconnection from the cell wall. It also undergoes condensation of chromatin and swelling of the endoplasmic reticulum. At the time this process is ongoing, vesicles housing sporopollenin precursors join with the plasma membrane, resulting in the release of their constituents into the locules of the anther, this is done for pollen wall construction. The tapetal cells secrete into the locule pollenkit and tryphine then the tapetum continues its degradation until it coats the pollen grains (Wilson and Zhang 2009).

3.3 Megasporangium

It is the ovary which houses the ovules. It can be both multi or uni-locular. The ovule rests on a free-central placenta that can be either parietal or axile. In some angiosperms such as *Butomopsis* the ovules occupy the inner walls of the ovary (Johri et al. 2001). Ovules or megasporangia remain are special in that they remain fused to their mother plants even after they are fertilized. This tight bond guarantees a plentiful nutrition for sporogenesis, the development of the gametophytes and the embryo formation (Bouman 1984; Wang 2010).

The ovules are comprised of three main elements. The nucellus which harbors the female gametophyte, a central chalaza which has integuments at its sides and the funiculus connecting to the carpel tissues. Ovule development begins when the subepidermal tissues of the placenta start dividing, this cell proliferation forms structures similar in shape to fingers that ultimately differentiate into an ovule (Boavida et al. 2005a). Ovules can be considered an aggregation of various sporangium-harbouring branches of which only one central branch remains reproductive and its peers divert collectively into an integument (Bouman 1984; Wang 2010). Ovules have one or two integuments. When they possess one they are named unitegmic, when two are integuments are presents they are called bitegmic. Bitegmy can be found in various monocots. Special cases occur in some *Oleaceae* where they lack integuments, this is called ategmy (Endress 2011a). Integuments are two layers of cells that form the seed coating. These layers coat the nucellus to form the micropyle which is a small aperture which the pollen tube penetrates during double fertilization. The curvature of the

ovule which gives it its usual shape is formed thanks to the asymmetrical growth of the outer integuments (Boavida et al. 2005a; Endress 2011b).

Most commonly fully matured ovules are classified into either anatropous, orthotropous, campylotropous or hemitropous types. This classification is based on the position of the micropyle relative to that of the funiculus. Anatropous where the micropyle lie close to the funiculus is the most common in angiosperms by far. The same is also true in basal angiosperms (Endress 2010).

3.4 Female Gametophyte

The female gametophyte is one of two alternating, morphologically and functionally different life styles of angiosperms, the other being the saprophyte. The female haploid gametophyte is responsible for generating haploid female gametes. These gametes then fuse with the male gametes and give rise to the zygote which begins the diploid sporophyte form (Yadegari and Drews 2004).

The female gametophyte which is embedded in the ovule pistil tissues is involved in many critical steps of the angiosperm reproductive process such as stigma-pollen recognition, pollen tube guidance, fertilization and the induction and control of seed development. Also called as the embryo sac or megagametophyte, this multicellular haploid organism develops within an ovule inside the carpel's ovary. The ovule is the site of female gametogenesis, fertilization, embryogenesis and seed production (Guo and Zheng 2013). The four different cell types that make up the female gametophyte play important roles in the reproductive process. Differentiation of these cells is tightly regulated and follows cues arranged along a polar axis, making the female gametophyte a remarkably versatile structure (Kägi and Groß-Hardt 2007).

For a successful fertilization to occur, the pollen tube must reach a small target, the female gametophyte which is within a large number of sporophytic cells. The pollen tube grows towards the ovule, against the surface of the funiculus reaching the micropyle so it penetrates the female gametophyte. It is necessary for the female gametophyte to send

directional signals and guide the pollen tubes. Data from physiological and genetic studies have shown that at least two such signals are produced, funicular guidance and micropylar guidance (Figure 2) (Higashiyama et al. 2003). These signals enable the pollen tube not only to navigate but also avoid abnormal female gametophytes (Ray 1997). The embryo sac hosts all the haploid maternal cells needed for the double fertilization, which occurs once the sperm cells reach the egg cell and central cell (Guo and Zheng 2013). Upon fertilization, genes expressed by the female gametophyte control the initiation of seed development (Chaudhury et al. 2001) while during seed development, gene products of the female gametophyte regulate endosperm and embryo development (Ray 1997).

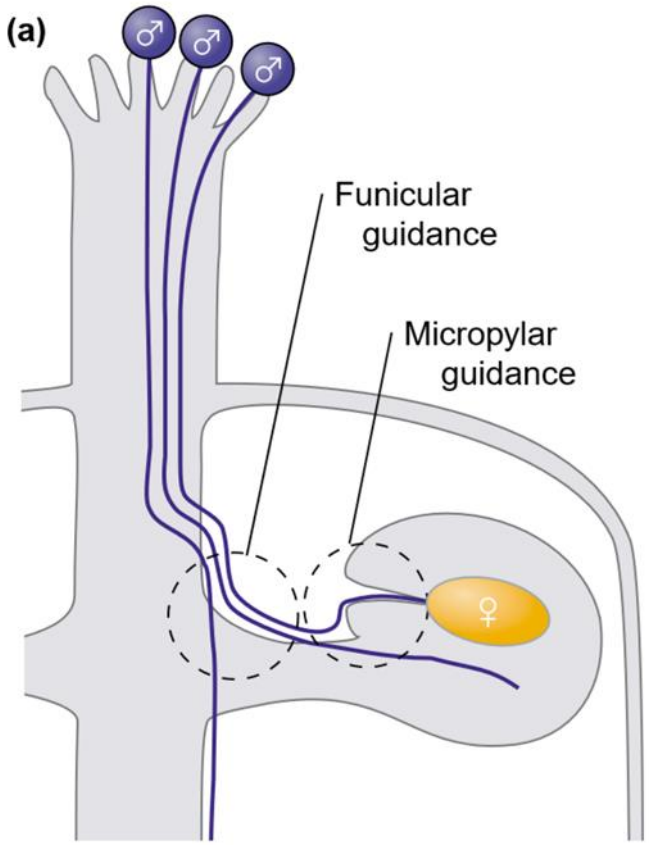

Figure 2: Pollen tube guidance in Arabidopsis thaliana.

Source: (Higashiyama et al. 2003).

In general, the female gametophyte development happens within the sporophytic ovule tissue which forms protective integuments around the gametophyte, in two distinct stages, namely megasporogenesis and megagametogenesis. More than a dozen different patterns of female gametophyte development have been described, differences arising from

variations in cytokinesis during meiosis, number and pattern of mitotic divisions, and pattern of cellularization. The development pattern of most flowering plants is the *Polygonum* type (Boavida et al. 2005a).

3.4.1 Megasporogenesis

Megasporogenesis is the developmental stage where the diploid megaspore mother cell generates four haploid spores via meiosis. Three main patterns of megasporogenesis is identified in angiosperms; monosporic, bisporic and tetrasporic represent differences in number of meiotic products contributing to the mature female gametophyte. In monosporic pattern, cell plate formation occurs after both meiotic divisions and four one-nucleate megaspores are formed. In the bisporic pattern the cell plates formation occurs, after meiosis I however before meiosis II begins, resulting in two two-nucleate megaspores. In tetrasporic pattern, no cell plates are formed, yielding one four-nucleate megaspore. Arising from these three different patterns is a single functional megaspore with one, two or four nuclei, while the other megaspores undergo cell death. The most common of the three patterns is the monosporic one which is represented in the polygonum pattern (Yadegari and Drews 2004).

3.4.2 Megagametogenesis

The next stage of development of the female gametophyte is megagametogenesis where the single functional surviving megaspore arising from megasporogenesis develops into the mature female gametophyte. First, the megaspore undergoes one or more rounds of incomplete mitotic divisions without cytokinesis forming a multinucleate coenocyte, followed by formation of cell walls around the multiple nuclei. This is the point at which a clear chalazal-micropylar polarity is established. In the common polygonum pattern, the megaspore undergoes two rounds of mitosis and gives rise to a four-nucleate cell with two nuclei at each pole. These sister and non-sister nuclei are then separated by phragmoplasts and cell plates forming in-between during a third round of mitosis. During this stage, the female gametophytes become completely surrounded by cell walls. Two nuclei, one from each pole, then migrate toward the center of the developing gametophyte and fuse together during cellularization. Cellularization yields a seven-celled structure with four distinct cell types; three antipodal cells, one central cell, two synergid cells and one egg cell (Yadegari and Drews 2004;

Boavida et al. 2005a). This structure may be further modified by cell death or proliferation in different species and represent distinct molecular and morphological attributes, with different functions in reproduction (Yadegari and Drews 2004)

The polarity establishment in female gametophyte development relates to the uneven development of the encompassing ovule layers. During cell differentiation, the two nuclei at the micropyle align in a different plane and develop into the egg cell, the micropylar polar nucleus and the synergid cells, while the two nuclei at the chalazal pole develop into the three antipodal cells and the chalazal polar nucleus. Additionally, in many species, the nucleus of the egg cell is located near the chalazal end and its vacuole occupies the micropylar end, while the synergid and central cells have the opposite polarity (Yadegari and Drews 2004; Boavida et al. 2005a).

A positional mechanism is likely to direct the establishment of cell fate during early female gametophyte development. Mutant analysis suggests that the female gametophyte polarity may be partly regulated by the surrounding sporophytic tissues. Previously thought to be a negligible influence, some cell-cell communication between the female gametophyte and the surrounding sporophyte has been reported Polarity establishment determines cell specification and ovule development and is important for cell morphogenesis and hormone gradients that guide development (Guo and Zheng 2013).

3.5 Male Gametophyte

Male gametophyte development requires a coordinated activity between different types of tissues and cells of gametophytic (pollen) and sporophytic (anther, tapetum) origin. Underlaid by their specific gene expression patterns. While it is a complex development process, compared to sporophyte, it is considered a highly reduced system (Honys et al. 2006). This reduction, with the protections which the saprophytic organs provide within the flower along with the strict selection of only the fittest pollen is the cause if the evolutionary success of angiosperm plants. These plants currently account for upwards of 280,000 species (Hafidh et al. 2016). The life cycle of the male gametophyte can be sorted into two developmental phases which are distinct. First is the developmental phase. It takes place within the locules of the anther and ends with the formation of mature pollen grains (Honys et al. 2006). Multicellular pollen is the progenitor cell of the male gametes and represent the male gametophytic generation of angiosperms, while unicellular pollen grains represent the microspores (Halbritter et al. 2018). Second, is the progamic phase which is initiated when mature pollen grains land on the stigma and ends at double fertilization (Hafidh et al. 2016).

3.5.1 Pollen maturation

Pollen development is initiated in the young locules of the anther. It consists of two sequential stages, microsporogenesis and microgametogenesis (Figure 3). The primary sporogenous layer produces diploid microsporocytes or meiocytes. Tetrads of four haploid microspores are given rise to by meiotic division. They are enveloped by a unique and thick callosic walls. The activity of the enzyme complex callase degrades the callose wall. This enzyme is secreted by the tapetum and leads to the separation of the tetrads into independent microspores. The development of the microspores is partnered with a vacuole biogenesis, fission and fussion events (Twell et al. 2006). The separated microspores enlarge and a lone vacuole is constructed. Simultaneously the microspore nucleus journey to a position against the microspore wall. The each of them undergoes an asymmetric mitosis (pollen mitosis I) (Borg et al. 2009). The asymmetry is caused by the dividing nucleus being peripheral to the wall and its orientation is such that when the cytokinesis is finished one cell is much larger than the other. The two bicellular pollen grain cells have completely deferent fates and structures (McCormick 2004). This asymmetry is essential, it allows for correct

cellular patterning of the male gametophyte, by reason of the two new cells harboring a distinct cytoplasm and they each possess a gene expression unique to them (Twell et al. 1998). The larger cell is vegetative, the smaller cell is generative (McCormick 2004). The smaller cell is engulfed by the cytoplasm of the large vegetative cell creating a cell within a cell structure. For this process to occur, the callose wall which separates the newly formed generative and vegetative cells must be degraded. After being fully engulfed the generative cell forms into a shape similar to a spindle. This shape is maintained thanks to a cortical cage made of bundled microtubes (Borg et al. 2009).

After pollen mitosis I, the larger vegetative cell exists the cell cycle at G1 after having dispersed the nuclear chromatin. The generative cell is nurtured by the vegetative cell which produces the pollen tube if a successful pollination occurs (Twell et al. 2006). During the pollen maturation, lipid and/or carbohydrate reserves are accumulated, along with proteins which are needed for rapid pollen tube growth by the vegetative cell (Pacini 1996). Osmotic protectants, including proline, sugars and glycine-betaine are also accumulated, they are believed to protect vital proteins and membranes from damage caused by dehydration. A state which pollen grains are in when released from the anthers (Schwacke et al. 1999). The smaller generative cell possesses a condensed nuclear chromatin (Twell et al. 2006). This cell continues and undergoes an additional round of mitosis, which is named pollen mitosis II so it can deliver twin sperm cells. In Arabidopsis thaliana and other species, which shed tricellular pollen, the pollen mitosis II transpires inside the pollen grain before the anthesis. However, the majority of plant species, for example *Lilium lonhiflorum* shed their pollen in a bicellular state and the pollen mitosis II happens inside the maturing pollen tube. After pollen mitosis II a male germ unit is established. It is a physical association among the vegetative nucleus and the sperm cells. If either the location or assembly of the male germ unit are affected by mutations will lead to reduced male transmission (Lalanne and Twell 2002).

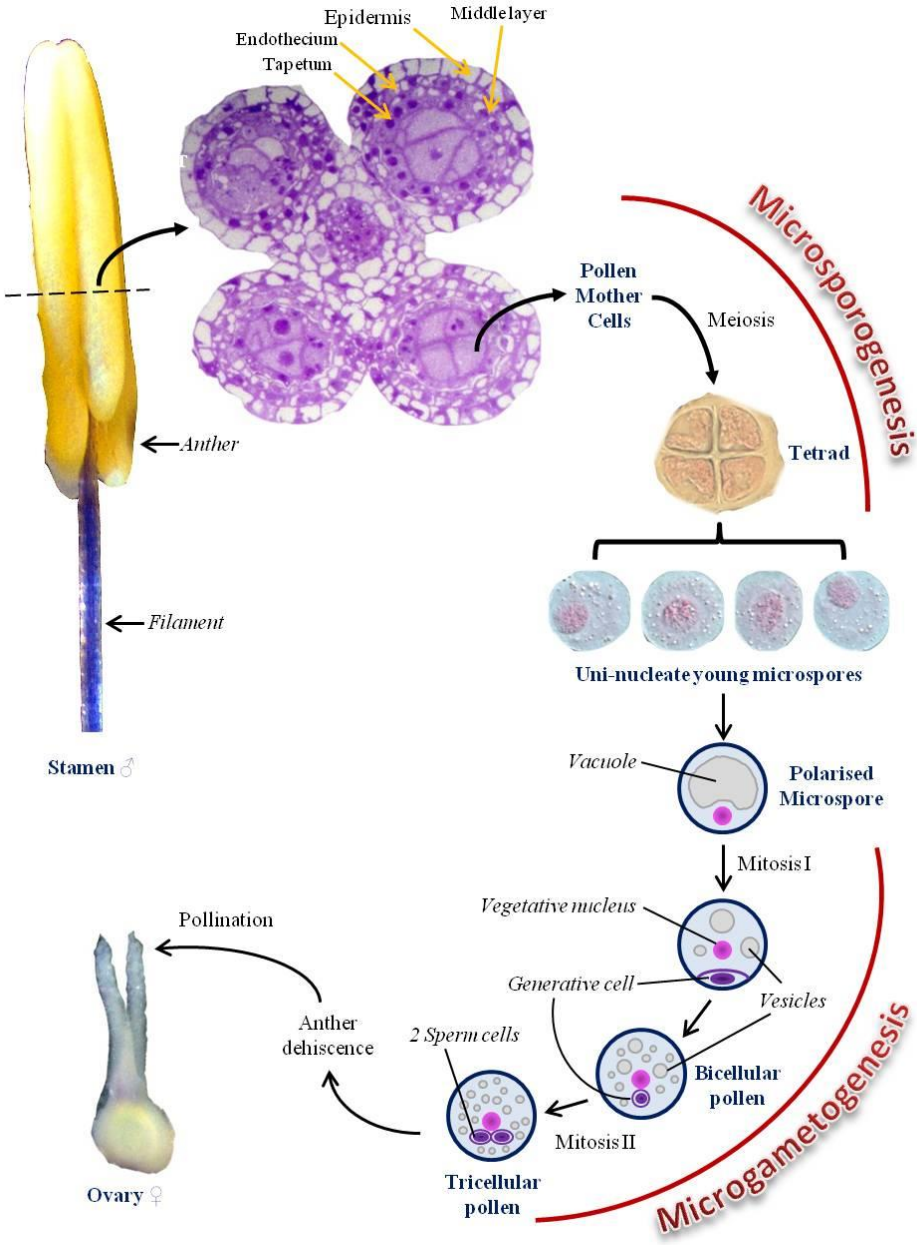

Figure 3: Development of the male gametophyte in angiosperms

Source: (Pacini and Dolferus 2016)

3.5.2 Pollen wall

The wall of the pollen grain is considered one of the most complex cell walls of plants (Shukla 1998). The main component of the pollen wall is sporopollenin, even though this material undergoes a slow autooxidation reaction, it is an extraordinary resistant wall biopolymer and is considered the strongest wall biopolymers produced by plants. This isolation and protection allow for the pollen grains to resist a majority of degrading

physiochemical agents. Pollen wall tend to be preserved in fossils even after all other biological structures have degraded in plant deposits (Heslop-Harrison 1968).

Pollen wall serves many indispensable functions such as resist desiccations of the male gametophyte which is exposed for prolonged periods of time to dry air during its hazardous journey to fertilize the female gametophyte. It acts as a barrier from UV light and provides general strengthening and protective function for the microspore. It acts as a repository for a number of allergens, exoenzymes and substances that recognize and determine intra- or interspecific reactions of incompatibility. It serves a role in pollen tube growth and germination (Shukla 1998) and facilitates a number of broad communications with the surface of the stigma (Scott et al. 2004).

Evolutionary, its development has been a key event in the development of the aquatic ancestors of the land plants. Its surface sculpturing has a high adaptability in relation to the pollen dispersal method (Shukla 1998). This sculpturing of the wall is precise and has unique patterning that it allows for pollen taxonomy and is a basis for identification of the different plant species (Heslop-Harrison 1968).

It is assumed that the formation of the pollen wall begins from meiosis, at the time of the callose which encloses the microspore starts a degradation process as a result of secretes originating from the tapetum. When the programmed cell deaths of the tapetum takes place, young microspores form primexine which is a microfibrillar matrix of cellulose (Shi et al. 2015). Primexine takes the role of a template and guide for the assembly of sporopollenin layer, the main component of the pollen wall. In case the callose wall is prematurely removed in microsporogenesis, a disruption in the pollen wall formation arises. Sporopollenin is polymerized by microspore precursors until the microspore is released, the majority of these sporopollenin precursors are secreted by the tapetum and are incorporated into the pollen wall only after the tetrad is dissolved (Scott et al. 2004). The formation of the wall is finished at the later stages of microgametogenesis where the remains of the degenerating tapetum settle and coat the pollen surface. The coat of the pollen is what determines its aroma, colour, adhesiveness and even taste. This serves vital function in facilitating recognition by insects and in the interactions between the pollen and papillary cells of the stigma (Hafidh et al. 2016).

The yellow and sometimes purple colour of the pollen grains is the results of both phenylpropanoid and carotenoid compounds accumulation (Honys et al. 2006).

The pollen wall is typically divided into three layers (Figure 4), which are the outer exine, the inner intine and tryphine or pollenkitt (pollen coat). Further the exine itself contains three layers; the reticulate layer, the ectexine layer (tectum and columella, foot layer) and finally a flat layer called the endexine. The ectexine is the layer which contains the taxon-specific sculpting. The ectexine functions as the skeleton of exine development. The main component of the exine is the sporopollenin which consist of phenolics and polyhydroxylated aliphatic compounds (Shi et al. 2015). Predetermined spaces in the exine which arise from an uneven distribution or lack of sporopollenin this gives rise to germinal apertures which are the regions for pollen tube emergence (Blackmore et al. 2007).

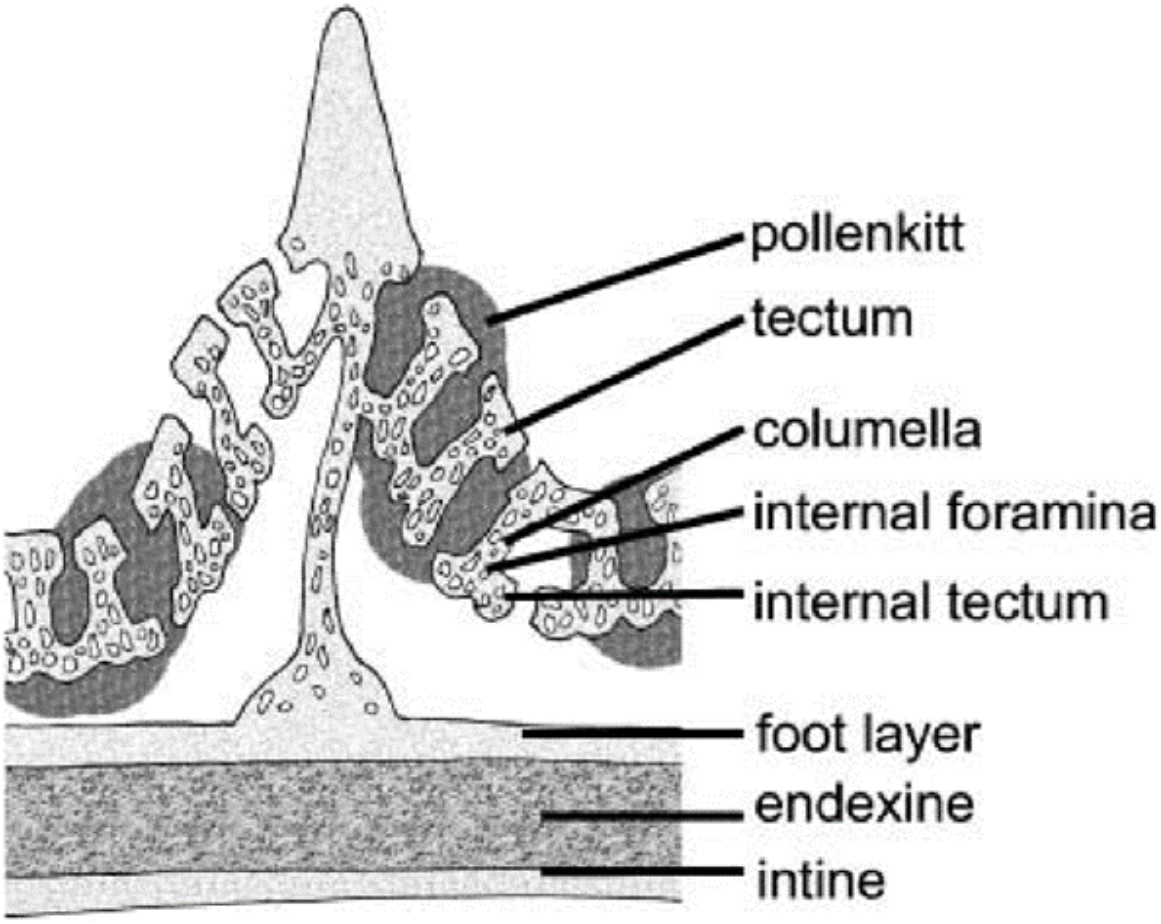

Figure 4: Pollen wall structure.
Source: (Blackmore et al. 2007).

3.6 Progamic phase

3.6.1 *In vitro* Pollen germination

In vitro pollen germination, while this method allows for a controlled system of experimentation, it does not completely replicate *in vivo* growth. The pollen tube growth can only achieve around 30% to 40% of its potential, in addition many structural anomalies are found in the pollen. *Arabidopsis thaliana* pollen does not germinate appropriately under *in vitro* conditions (Taylor and Helper 1997). *In vitro* germination of pollen in most plant species is achievable by placing it in a solution of boron, a source of carbohydrates such as typically used sucrose and calcium (Patel and Mankad 2014). Germination medium composition has a large effect on pollen germination and tube growth (Taylor and Helper 1997).

Sucrose serves an additional function besides providing energy, specifically it is an osmoticum. It takes the role of a substrate for the metabolism of pollen and maintains the osmotic pressure (Patel and Mankad 2014). In some plants, sucrose is a poor osmotic regulant, such as rye, wheat and eggplant pollen, here a better alternative is maltose (Jayaprakash 2018). High levels of sucrose in germination medium can result in a change of the pollen tubes permeability, which culminates in the pollen grain draining ions and metabolites into the germination medium. A danger of sucrose is its fermentation reaction into ethanol which if allowed to accumulate can result in an inhibited growth (Taylor and Helper 1997). Boron facilitates pollen germination by the means of H^+-ATPase activity which is responsible for initiating the germination and tube growth activity. Generally, as pollen grains contain already plenty of vitamins, hormones and amino acids there is no need to supply the medium with them. There are however some studies which suggest auxins, cytokinins and gibberellins in small concentrations are positive for pollen germination and tube growth (Patel and Mankad 2014). It was found that flavonoids have stimulant effects on pollen germination and tube growth by observing plants deficient in flavanol which are self-sterile because of the pollens inability to germinate. This sterility can be reversed if flavanols at the time of pollination are used (Taylor and Helper 1997).

Various environmental factors affect both the processes of pollen germination and tube growth in the male gametophyte and their interaction with the female gametophyte as in adhesion of pollen, pollen tube guidance and fertilization. There is a variation among spices

in the temperature tolerance of pollen germination and tube growth. Plants from warmer climates were found to be able to tolerate temperature from 10 °C and above. In plants originating in the temperate zones 5 to 10 °C were found to be tolerable. High mountain plants pollen germination and tube growth was able to function even in 0 °C (Wagner et al. 2016).

3.6.2 Pollen germination

Pollen adhesion is the first occurrence of postpollination. It is an event dependent on the properties of the stigma surface and pollen. The stigma is the one that provides any lacking materials in pollen needed for germination. Adhesion is critical in stigmas with dry surface and germination needs to depend on the pollen coat components. The process is much easier in stigmas with wet surfaces where the process is not critical (Shivanna et al. 1997), they secrete a sticky substance named the exudate. Tobacco is a plant possessing what we call wet stigmas, their exudate secrete is involved not only in pollen recognition and capture but also in control of the water content during hydrazination process. The exudate is composed of phenols, glycoproteins, proteins, polysaccharides, enzymes and lipids which are made of unsaturated and saturated fatty acids of triacyl-glycerides, there are other minor components (Boavida et al. 2005b). Following the adhesion, pollen coat is mobilized and form lipid-protein foot on the stigma surface (Edlund et al. 2004).

Pollen grains are dehydrated when released from the anthers, averaging a water content range 15 to 35 %. After pollen lands and attaches on a compatible stigma a rapid process of hydration takes place. Nutrients, water and other substances flow into the pollen grains through germination apertures (Edlund et al. 2004). The process is accompanied by a massive discharge of amino acids and the formation of vacuoles, vesicles and lipid droplets. In addition, ribosomes encrust the endoplasmic reticulum increasing raspatory activities of the mitochondria (Honys et al. 2006). Lectins are proteins which interact with carbohydrates are present in the pollen cell wall, they facilitate control in intracellular transport and recognition processes. It has been shown that after a brief incubation tobacco pollen grains exude proteins including lectins. These lectins have been found to have a positive effect on pollen germination *in vitro* (Matveeva et al. 2007).

Hydration process is well regulated. The water is extracted using metric potential at first, then after the plasm membrane is formed a systems of turgor pressure differentials. A balance is formed between the hydraulic pull the pollen exerts and the resistance by stigma surface (Heslop-Harrison 1992). Aquaporins are theorized to be involved in the water flow after the changes in the membrane architecture occur (Honys et al. 2006).

The changes in water content may act as an initiation signal for the pollen germination. (Shi and Yang 2010). The pollen is transformed from a nonpolar cell to a polarized. The pollen must reorganize its cytoplasm and cytoskeleton for the tube to be able to emerge. This includes the creation of filamentous cytoskeleton structures which envelop the nuclei and actin cytoskeletal polarization in the site of the tube growth (Edlund et al. 2004). The abundance of the actin modulating proteins suggest a great importance of the polymer in germination and tube growth. Treatment of pollen *in vitro* with actin inhibitors have greatly reduced germination rate and pollen tube growth. The inhibitory effect was more prominent in tube elongation possibly because the presence of actin filaments is critical in tube growth but less so in germination, other possibility is that germination is less susceptible to depolymerization of the actin cytoskeleton (Gibbon et al. 1999). The reorganization is triggered by the influx of Ca^{2+}. Its influx causes an accumulation of cytoplasmic gradient and vesicles underneath the pore close to the adhesion site. The germination of the pollen grain leads to the pollen tube growth at the site of the aperture, if the grain is unapertured the tube can rise from any place (Feijó et al. 1995; Shi and Yang 2010).

Proteomic research on *Arabidopsis* suggest that pollen grains have a pre-synthesized number of proteins necessary for germination and tube elongation, most significantly they have stored proteins related to translation and the ribosome. This is done to facilitate a quick process of germination and tube growth (Dai et al. 2007).

3.6.3 Pollen tube growth

Pollen tube growth while not isodiametric represents a type of tip growth similar to hyphae and root hair. This growth is defined by its continuance and fast elongation at the cell tip without additional divisions of the vegetative cell. Pollen tubes have been observed to reach a maximum length of 50 cm. The tube forms callose plugs in set distances to maintain a stable amount of cytoplasm (Hafidh et al. 2016)

For growth is narrowed to the tube tip (apex) where new material is deposited, its cell wall is deformable. It also undergoes new, highly dynamic membrane and cell wall material integration. In the distal parts of the tube it is necessary for it to be more static since for it must resist turgor. The apex of pollen tubes is typically comprised of a sole layer of methyl esterified and acid pectins. A small distance behind the apex in the subapical zone the pectins are de-esterified and a small amount of cellulose are present in the wall. The tubular portions are mainly made of callose (Vogler et al. 2013).

Pectins are the major carbohydrate polymers of the tube wall. The biosynthesis of different pectin motifs is realized in the Golgi apparatus and are secreted by Golgi-derived vesicles. These vesicles transport pectins and other materials migrating toward the tip where they accumulate and fuse with the plasma membrane to sustain the tube growth forming the extending cell wall (Mollet et al. 2013). The actin cytoskeleton is necessary in the transport of secretory vesicles as they use it as a guide toward the apical region (Zheng et al. 2018). The cytoskeleton the same as in eukaryotic cells includes microtubules and microfilaments (Steer and Steer 1989). Periodic growth of actin bundles into the apex transpires between the exocytosis of materials transported by the vesicles (Honys et al. 2006).

Once the pectins are fused into the cell wall, they are demethylated exposing negatively charged carboxyl groups and releasing protons (H^+). A lower intracytoplasmic pH is established. The free carboxyl groups bind to the Ca^{2+} and form interchain bridges. Most of the processes at the apical zone are related to the localized calcium gradient. The concentration of Ca^{2+} is larger in the apex, this is the result of the asymmetric activity of Ca^{2+} pumps and channels which are present in the pollen tube plasma membrane. These channels are activated by either signal transduction or mechanical systems. The calcium gradient is

indispensable for the elongation process and its disruption causes an inhibition of the growth rate (Parrotta et al. 2019). The ion fluxes and gradients are oscillatory this is reflected in the pollen tube growth (Hafidh et al. 2016).

Growth of the pollen tube alternates between two periods, one of activity and the other of inactivity. The growth itself is thanks to the work of two systems. The first ensures a balance between the forces of internal pressure (osmotic and turgor) and the deformation resisting force of the cell wall. The second system is responsible for the ion dynamics and internal proteins which lead to materials settling at the growth tip by vesicles (Tambo et al. 2016). It is by overcoming the resistance of the pollen tube wall that elongation is and penetration into the pistil is possible (Parrotta et al. 2019).

Findings suggest that the mechanical properties of the pollen tube wall are similar to that of rubber. This enables a pollen tube that is fully turgid to be both stiff and flexible. The tube is able to quickly react to obstacles in its path and attractants by modifying its path of growth (Vogler et al. 2013).

Cytoplasmic streaming happens in cell that are cylindrical in shape and growing. It involves four activities. Organelles, cytoplasmic inclusions and lipid bodies circulate the tube length. Many precursors which contain polysaccharides fuse into the growing tip wall. The vegetative nucleus migrates forward. Passage of the generative cell forward (Honys et al. 2006).

The pollen tube has a two layered cell wall and is divided into four different zones (Figure 5), the apical zone, the subapical zone, nuclear zone and the vascular zone (Malhó and Salomé 1992). The apical zone which is the growing zone is enriched with both types of vesicles (secretory and smaller). Subapical zones cytoplasm houses organelles, mitochondria, plastids (amyloplasts), endoplasmic reticulum (ER), lipidic bodies and especially active dictyosomes are numerous. Nuclear zone contains both the vegetative and generative nuclei. Vacuolization zone contains large vacuoles separated by the callose plugs (Cresti et al. 1977).

The increase in the tube volume correlates with an increase of the vascular zone while a decrease is observed in the volume occupied by the vegetative nucleus, generative cell and mitochondria suggests that these organelles move to tip during tube growth (Malhó and Salomé 1992).

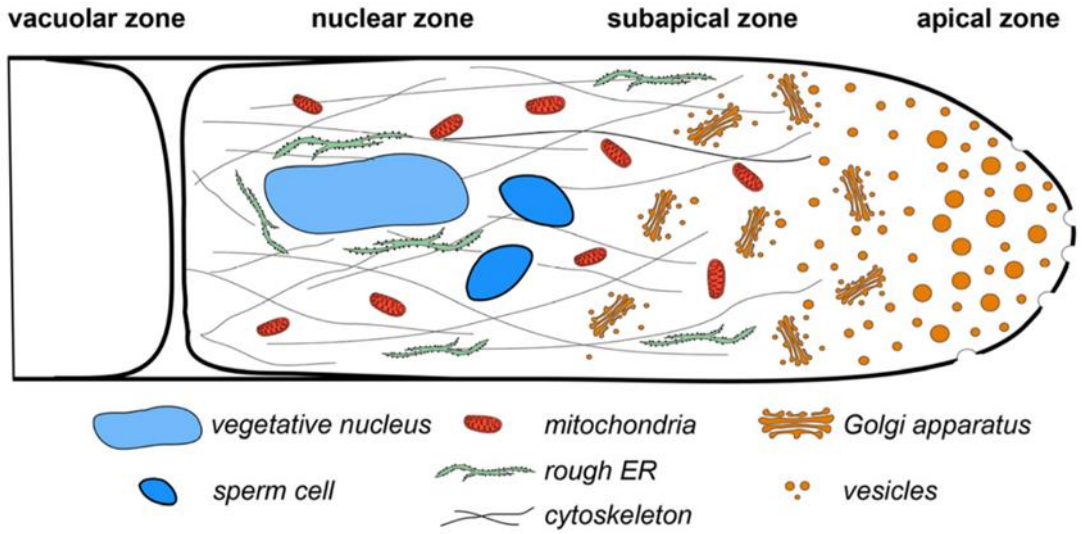

Figure 5: Pollen tube regions
Source: (Honys et al. 2006).

3.7 Atonik

Biotic and abiotic stress affect plants in a negative manner, causing decreases in crop yields. To contain these loses, biologically active substances or biostimulants may be used, these substances can help reduce yield loses by supporting the natural resistance of the plants. Biostimulants can be sorted according to their origin into two groups: Ntural which are based on extracts from fruits or seaweeds, amino acids, chitosan, humic substances or effective microorganisms and synthetic which consist of salts, growth regulators, vital elements and phenolics. Counted among synthetic biostimulants is Atonik also known as Asahi SL in Poland or Chaperone in USA (Kocira et al. 2017).

Atonik is a commercial product manufactured by the Japanese company Asahi Co., Ltd. Its active ingredients are 0.1% sodium 5-nitroguaiacolate ($NaC_7H_6NO_4$), 0.2% sodium 2-nitrophenolate ($NaC_6H_4NO_3$) and 3% sodium 4-nitrophenolate ($NaC_6H_4NO_3$) and water. The

active ingredients are called nitrophenolates. Atonik is used in over 20 countries and has been registered for use as a pesticide in soybeans, cotton and rice. It has been marketed as a protein transport enhancer for cotton (Djanaguiraman et al. 2010)

Atonik is able to increase the rate in the cells plasma streaming and stimulate the plant metabolic activity without inducing toxicity or malformations. It increases the concentrations of auxins in the plants (Djanaguiraman et al. 2004), this is achieved by an increase in the inhibition of indole-3-acetic acid oxidase which causes a higher activity of naturally occurring auxins. An increase in nutrient uptake and assimilation has been found in plants treated with Atonik caused by the auxin increase which enhances the shoot and root elongation resulting in a higher seedling biomass and faster development (Djanaguiraman et al. 2005a). An increase in photosynthesis in plants prayed with Atonik was also found. These plants had a higher leaf area, photosynthetic intensity, chlorophyll a fluorescence and overall chlorophyll content. In preliminary research an increased leaf area index in wheat plants was found. Atonik increased the transpiration rate without reducing the relative water content. These positive effects are more visible when applied to plants under stress, leading to Atonik being used protectively in plants exposed to adverse temperatures, high metality or salinity and even drought. However, it could be said that with no such stress conditions the positive effects might not be noticeable (Przybysz et al. 2014).

Research has been conducted for the usage of Atonik against diseases. A spray of Atonik at weekly intervals for four times significantly decreased the spread of *Puccinia antirrhini* on snapdragon, hindered *Melampsora epitea* development on willow and *Erysiphe biocellata* on Mentha (Wojdyła 2004).

4 Methodology

The research was relegalized in the laboratories of the Institute of experimental botany of the Czech academy of sciences.

4.1 Plants material

Pollen from wild type tobacco plants (*Nicotiana tabacum* cv. Samsun) were used in this study. Tobacco seeds were planted in a greenhouse. Conditions were short-day, and temperature was kept between 22 to 25 °C. When the plants mature and have fully formed roots, they are relocated to an outdoor greenhouse with on ground compost. They were grown under natural conditions of day-night photoperiod during spring and summer.

Flowers were removed from plants during the growing season, in dry weather conditions. Only flowers with pink crown tips, measuring around 52 to 55 mm just before floral opening are collected. Opened flowers were collected too if the anthers were intact. Stamens were removed from the flowers into a watch glass or petri dishes and left to dehisce overnight. Pollen grains where than poured from the dry anthers and filtered through nylon mesh with a pore size of 50 µm to remove anther debris. Pollen is then collected, weighted and placed in Eppendorf Tubes®. The tubes are then stored under -20 °C.

4.2 *In vitro* pollen cultivation

SMM-MES cultivation medium was used. Murashige and Skoog basal medium powder from Sigma-Aldrich was mixed with the following substances:

- Sucrose 0,175mM
- H_3BO_3 1,6mM
- $Ca(NO_3)_2.4H_2O$ 3mM
- $MgSO_4.7H_2O$ 0,8mM
- KNO_3 1mM
- MES monohydrate 23mM

The cultivation medium must have a pH of 5,9, pH is adjusted as necessary using potassium hydroxide, after which the medium is twice (one day interval) sterilized in a boiling water pot. The medium is prepared in bulk and stored inside of jars and kept in a cold room for later use.

One litre bottle of the biostimulator Atonik in a soluble concentrate form was procured online. The bottle contained the following active ingredients: 1 g/L sodium 5-nitroguaiacolate, 2 g/L sodium 2-nitrophenolate and 3 g/L sodium 4-nitrophenolate.

The SMM-MES medium was placed in a 30 °C water bath to be tempered before use. Pollen was left at room temperature for 10 minutes also before use. 20 mg of pollen was resuspended into 10 ml of SMM-MES medium for the control variant and into 10 ml of SMM-MES with Atonik in 0.1%, 0.2%, 0,5% concentrations for variants B, C and D respectively. This was done in a sterile laminar flow cabinet.

Erlenmeyer flask were used for the pollen cultivation, each flask contained 10 ml of medium (with the appropriate concentration of Atonik) and 20 mg of pollen. The pollen cultures were then placed into a water-bath shaker at 140 rpm for 3 hours and then slowed down to 90 rpm for the remaining time, with no light conditions.

Pollen germination and tube growth was tested at different concentrations of Atonik (control, 0.1%, 0.2% and 0.5%) and for three different time durations (1 hour, 2 hours and 4 hours). Each test was repeated three time.

4.3 Analysis

Microscopy was carried out using Nikon TE2000-E inverted microscope. Images of the pollen cultures were captured at x4 magnification with 0.84 µm/pixel using NIS-Elements AR 3.0 software. Data collection from the images of the pollen cultures was done using freely available ImageJ software. At least 50 pollen tubes were measured for each test repeat.

Pollen was judged as germinated if it had a visible protruding tube tip longer than 4 µm. Germination was calculated as:

$$\text{Germinated pollen grains/Total number of pollen grains} \times 100 = \text{Germination rate}$$

A normal distribution of data was assumed. One-way ANOVA with Scheffé's test was performed using Microsoft Excel 2016 and website www.astatsa.com .

5 Results

5.1 Pollen tube growth and germination after 1 hour of cultivation

Pollen tube length were successfully measured after cultivation for 1 hour under 10 °C. The longest tubes were observed in variant with 0.1% Atonik treatment (B) with a mean length of 9.245 µm (Table 1) followed by 0.2% Atonik treatment (C) which achieved 8.224 µm. Shortest pollen tubes were in the control variant (A) with 7.087 µm. Variant B had a statistically significant difference with variant A while no difference was between it and variant C. This suggests a positive effect of 0.1% Atonik in the 1. hour under 10 °C.

Under 15 °C cultivation variant C had the longest pollen tubes at 26.265 µm, only a small difference was between variants A 23.000 µm and B 22.872 µm. There was no statistically significant deference between the variants. This shows that 0.1% and 0.2% Atonik have no effect on pollen tube length under 15 °C in the 1. hour.

At 18 °C the longest pollen tubes were found in the A variant at 27.586 µm followed by B variant at 26.555 µm, C variant had a mean of 23.834 µm. There was no statistically significant difference between the variants, this is similar to the results under 15 °C, showing no statistically provable effect of 0.1% and 0.2% Atonik on pollen tube length.

Cultivation at 26 °C yielded longest tubes in B variant at 54.991 µm, C variant had a mean of 48.334 µm and variant A had a mean of 44.302%. The shortest pollen tubes were in variant with 0.5% Atonik (D) at 26.618 µm. There was no statistically significant difference between variants A, B and C however there was a significant difference between them and variant D, demonstrating an inhibitory effect of 0.5% Atonik on pollen tube length.

Under 30 °C longest pollen tubes were observed in variant C at 90.332 µm, shortest pollen tubes were in variant D. Statistically provable difference between variant C and variant A which had a mean of 69.882 µm. This suggests 0.2% Atonik has positive effects on pollen tube length during the first hour under 30 °C. Variant D tube length was significantly lower than other variants confirming the inhibitory effect of 0.5% Atonik.

Table 1: Pollen tube length after 1 hour of cultivation.

Treatment		**Temperature**									
		10 °C		15 °C		18 °C		26 °C		30 °C	
		Mean $\bar{x}$	*SD*	*Mean* $\bar{x}$	*SD*	*Mean* $\bar{x}$	*SD*	*Mean* $\bar{x}$	*SD*	*Mean* $\bar{x}$	*SD*
A	Control	7.087^{a*}	4.155	23.000^{a}	21.784	27.586^{a}	26.113	44.302^{a}	47.382	69.882^{a}	65.042
B	0.1% Atonik	9.245^{b*}	7.383	22.872^{a}	20.550	26.555^{a}	26.047	54.991^{a}	51.721	84.923^{ab}	79.723
C	0.2% Atonik	8.224^{ab}	5.536	26.265^{a}	25.922	23.834^{a}	25.032	48.334^{a}	56.158	90.332^{b}	75.785
D	0.5% Atonik	0	0	0	0	0	0	26.618^{b*}	23.614	46.307^{c*}	44.362

Note: Measured in µm, x4 magnification. Means with different subscripts have a significant difference ($p < 0.05$), means with * differ ($p < 0.01$)

Under 10 °C most germinated pollen was found in B variant at 16.62% (Table 2). A variant had 14.45% germination rate while variant C germinated at 9.70%. No statistically significant difference was found between variant A compared to B and C however a significant difference was found between B and C. In variant D no pollen grains germinated (Figure 6) confirming an inhibitory effect of 0.5% Atonik on pollen germination

At 15 °C pollen grains in variant B achieved the best germination rate at 57.39%, followed by variant A at 57.39% and variant C at 45.42%. No statistically significant difference was found in variant A compared to variants B and C. Between variants B and C there was found a provable statistical difference. Variant D did not germinate.

Pollen grain germination rate was similar with no statistically significant difference under 18 °C between the variants A 49.21%, B 51.15% and C 52.19%. Variant D did not germinate (Figure 7) at all as in variants cultivated under 10 °C and 15 °C.

Pollen grains cultivated under 26 °C germinated nearly at the same rate in in variant B at 56.71% and A at 56.65%. Variant C germinated at 53.28%. The difference between the three variants was statistically insignificant. Variant D had the worst germination rate at 15.10% with a statistically significant difference than the other variants.

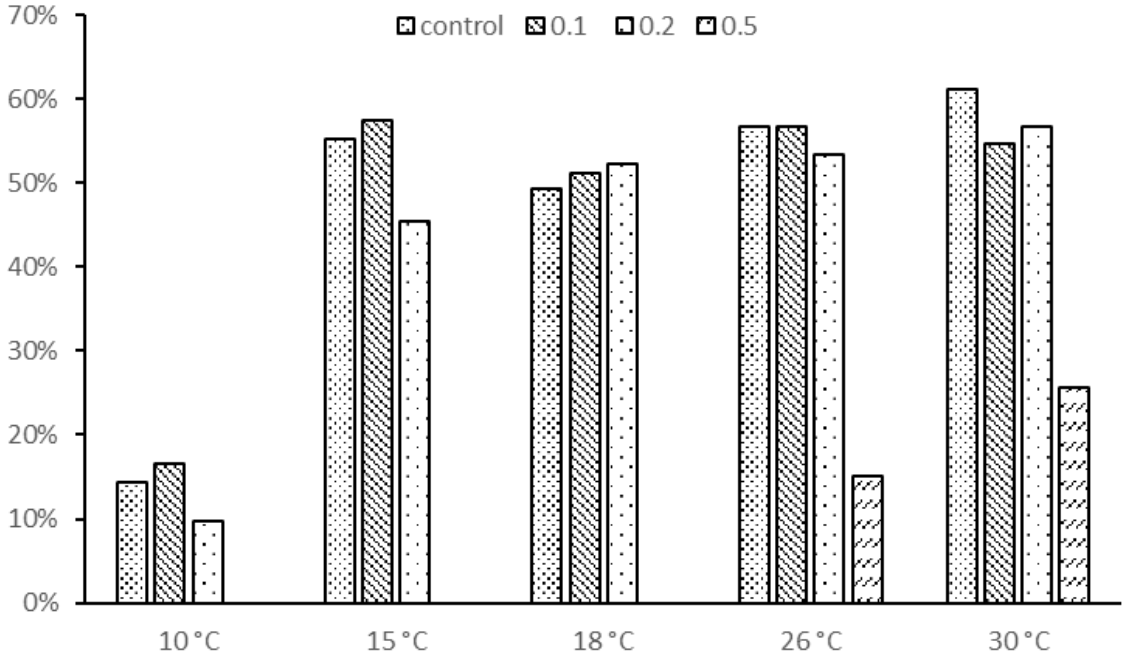

Figure 6: Pollen germination after 1 hour of cultivation.

Table 2: Pollen germination percentage after 1 hour of cultivation.

Treatment		Temperature									
		10 °C		15 °C		18 °C		26 °C		30 °C	
		Mean $\bar{x}$	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*
A	Control	14.45^{ab}%	3.77	55.19^{ab}%	7.16	49.21^{a}%	9.83	56.65^{a}%	6.53	61.17^{a}%	16.29
B	0.1% Atonik	16.62^{a}%	3.79	57.39^{a}%	5.49	51.15^{a}%	15.63	56.71^{a}%	11.22	54.69^{a}%	14.77
C	0.2% Atonik	9.70^{b}*%	2.99	45.42^{b}%	8.56	52.19^{a}%	13.92	53.28^{a}%	6.27	56.75^{a}%	8.23
D	0.5% Atonik	0	0	0	0	0	0	15.10^{b}*%	6.62	25.64^{b}*%	10.62

Note: Means with different subscripts have a significant difference ($p < 0.05$), means with * differ ($p < 0.01$).

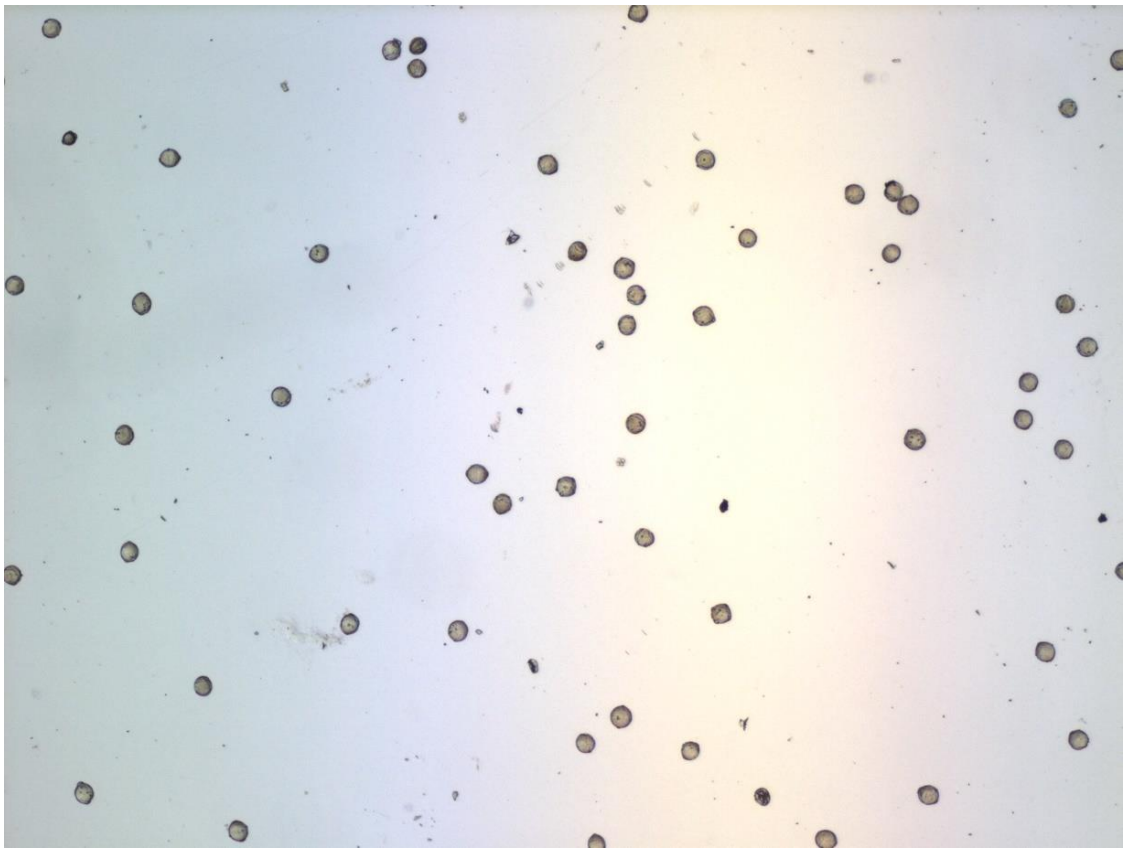

Figure 7: Pollen grains cultivated under 18 °C with 0.5% Atonik treatment after 1 hour.

5.2 Pollen tube growth and germination after 2 hours of cultivation

The three variants A, B and C cultivated for 2 hours under 10 °C were statistically indifferent with close mean lengths (Table 3). Variant C had a mean length of 14.712 µm, variant A 14.525 µm and variant B 13.411 µm.

At 15 °C cultivation best result was found in variant A with 72.835 µm mean length followed by variant A at 58.556 µm and variant B at 57.083 µm. The difference between A to B and A to C was statistically insignificant. Variant B had approvable statistically difference to variant C.

Under 18 °C pollen tube length was similar in variant C at 67.888 µm and A at 67.383 µm. The differences between the three variants A, B and C were statistically insignificant. Worst pollen tube length was in variant D at 35.581 µm with a statistically significant difference to variant A, confirming an inhibitory effect of 0.5% Atonik treatment.

Longest pollen tubes in 26 °C cultivation was in variant A (Figure 8) at 190.402 µm. There was no statistically significant difference between the variants A, B 157.179 µm and C 175.297 µm. Shortest pollen tubes were found in variant D 80.084 µm with a significant statistical difference to the other three variants.

Pollen grains under 30 °C grew the longest pollen tubes in variant B at 302.291 µm. Variant C had a mean length of 283.455 µm and A had a length of 265.564 µm. No statistically significant difference was found between the three variants. The worst pollen tube elongation was found in variant D at 107.715 µm with a statistically significant difference to the other three variants.

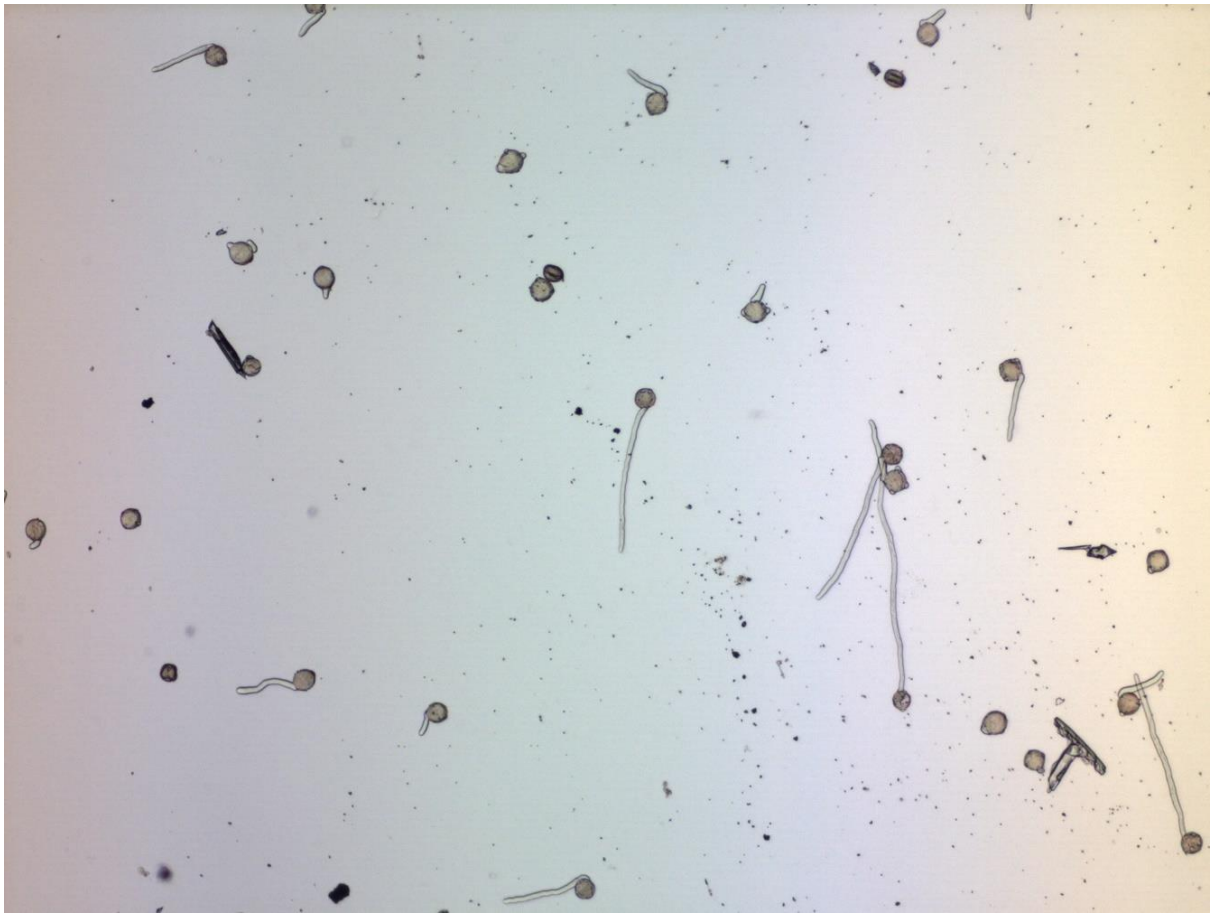

Figure 8: Pollen cultivated under 26 °C after 2 hours.

Table 3: Pollen tube length after 2 hours of cultivation.

Treatment		Temperature									
		10 °C		15 °C		18 °C		26 °C		30 °C	
		Mean $\bar{x}$	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*
A	Control	14.525^{a}	15.500	58.556^{ab}	46.923	67.383^{a}	59.235	190.402^{a}	133.834	265.564^{a}	153.842
B	0.1% Atonik	13.411^{a}	15.021	72.835^{a}	68.517	54.833^{ab}	64.624	157.179^{a}	149.107	302.291^{a}	203.477
C	0.2% Atonik	14.712^{a}	18.668	57.083^{b}	59.624	67.888^{a}	76.092	175.297^{a}	147.45	283.455^{a}	212.011
D	0.5% Atonik	0	0	0	0	35.581^{b*}	42.511	80.084^{b*}	81.447	107.715^{b*}	97.273

Note: Measured in µm, x4 magnification. Means with different subscripts have a significant difference ($p < 0.05$), means with * differ ($p < 0.01$)

Under 10 °C best pollen germination was found in variant A 48.80% (Table 4) followed by variant B 43.01%, difference between both variants was statistically insignificant. Variant C had a germination of 31.91% and had a statistically provable difference to variant A. Showing an inhibitory effect of 0.2% Atonik under this temperature. Variant D did not germinate (Figure 9).

At 15 °C there was no statistically significant difference found between the three variants B 61.27%, A 59.26% and C 51.41%. Variant D did not germinate, the same result found in the 10 °C cultivation conditions.

Pollen germinated at similar rates under 18 °C. Variant A had a rate of 64.34%, followed by variant B at 61.24% and variant C at 60.78%. No statistically significant difference was found between these variants. However, variant D started to germinate after 2 hours (Figure 10) at a rate of 12.94%, this was the worst rate in the group with a significant statistical difference compared to the other variants.

The germination rate of pollen grains under 26 °C was similar with no significant statistical difference in the three variants. Variant A had a rate of 63.70%, variant B a rate of 56.94% and C 53.80%. Worst germination rate was in variant D at a rate of 27.03% with a significant statistical difference compared to the other three variants.

At 30 °C pollen germinated at a rate of 74.76%, variant B at 70.22% and C at 68.45%. No statistically significant difference was found between the variants. Worst germination rate was found in variant D at 41.38% with a statistically significant difference compared to the variants A, B and C.

Table 4: Pollen germination percentage after 2 hours of cultivation.

Treatment		Temperature									
		10 °C		15 °C		18 °C		26 °C		30 °C	
		Mean $\bar{x}$	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*
A	Control	47.80^{a}%	11.60	59.26^{a}%	11.96	64.34^{a}%	6.19	63.70^{a}%	14.54	74.76^{a}%	13.37
B	0.1% Atonik	43.01^{ab}%	11.83	61.27^{a}%	11.89	61.24^{a}%	6.21	56.94^{a}%	14.11	70.22^{a}%	6.42
C	0.2% Atonik	31.91^{b}%	3.88	51.41^{a}%	10.85	60.78^{a}%	8.00	53.80^{a}%	14.68	68.45^{a}%	7.78
D	0.5% Atonik	0	0	0	0	12.94^{b}*%	20.7	27.03^{b}*%	9.40	41.38^{b}*%	9.71

Note: Means with different subscripts have a significant difference ($p < 0.05$), means with * differ ($p < 0.01$).

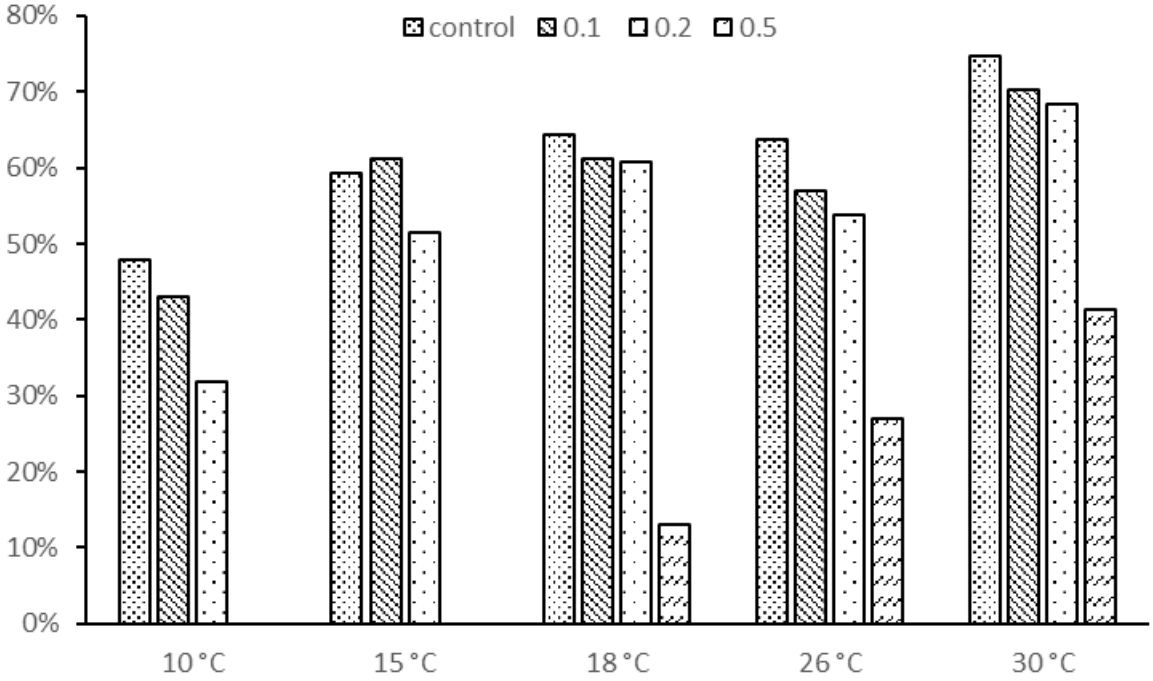

Figure 9: Pollen germination after 2 hours of cultivation.

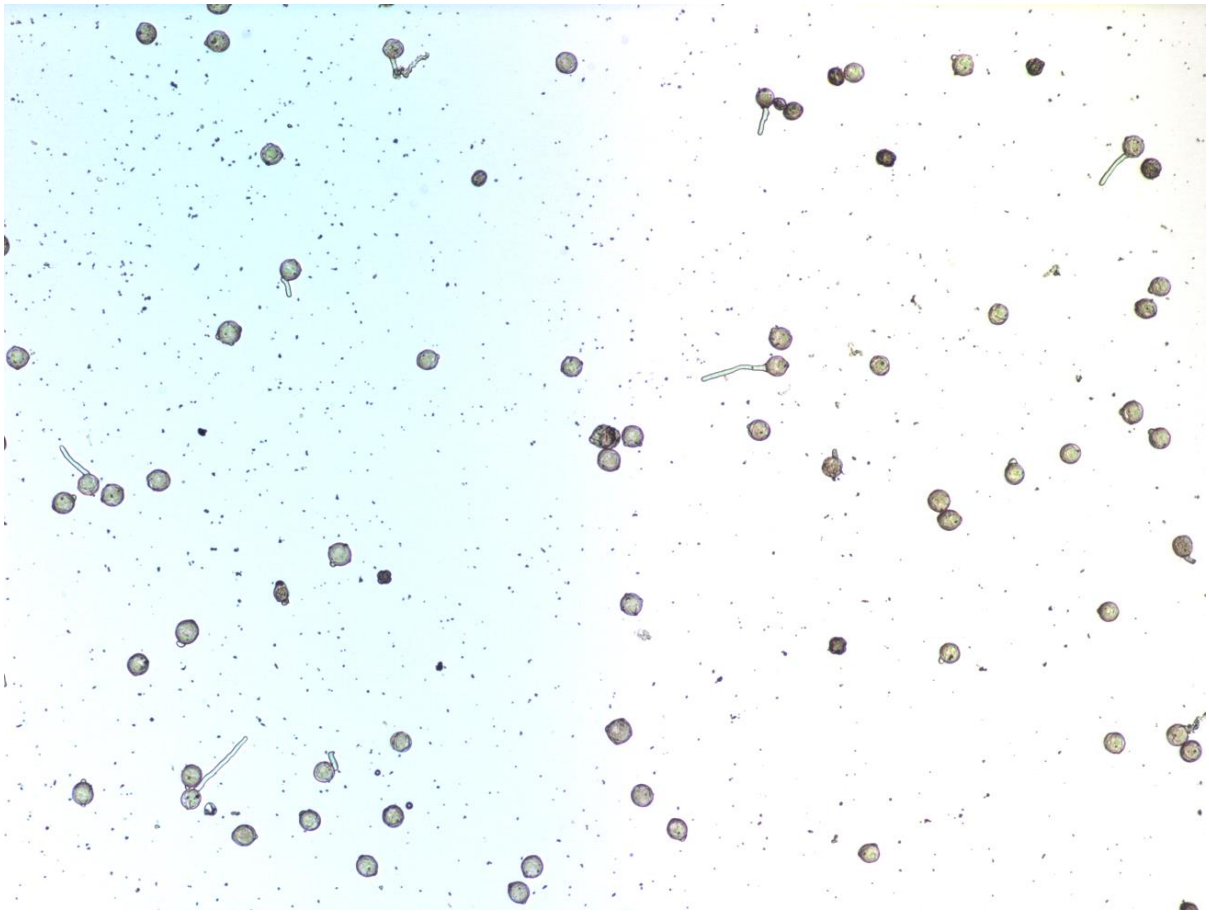

Figure 10: Pollen cultivated under 18 °C after 2 hours.

5.3 Pollen tube growth and germination after 4 hours of cultivation

Under 10 °C the longest pollen tubes were found in A variant at 33.945 µm (Table 5), no significant statistical difference was found between A variant and B variant 26.707 µm. C variant had tube lengths of 21.079 µm with a significant statistical difference compared to variant A.

At 15 °C pollen tubes of variant A were the longest at 173.932 µ, followed by variant B at 157.696 µm and variant C at 154.476 µm. However no statistically provable difference was found between these three variants.

Pollen grains grown under 18 °C formed longest tube in variant A at 282.462 µm, variant B had a mean of 262.022 µm and variant C a mean of 254.805 µm. The three variants had a statistically insignificant difference between them. Variant D had the shortest pollen tubes at 79.620 µm with a significant statistical difference when compared to variants A, B and C.

Longest pollen tubes under 26 °C were observed in variant A 606.738 µm, however no significant statistical difference was found between it and varaints B 594.685 µm and C 576.037 µm. Shortest pollen tubes were again found in variant D at 255.575 µm with a statistically significant difference compared to the other variants.

Pollen cultivation at 30 °C yielded a statistically insignificant difference mean lengths between the variants A 636.731 µm, B 656.700 µm and C 606.859 µm. Variant D was similar to the previous results with the shortest tubes at 384.024 µm with a statistically significant difference compared to the other variants. This confirms an inhibitory effect of 0.5% Atonik on tube elongation.

Table 5: Pollen tube length after 4 hours of cultivation.

Treatment		Temperature									
		10 °C		15 °C		18 °C		26 °C		30 °C	
		Mean $\bar{x}$	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*
A	Control	33.945[a]	29.134	173.932[a]	112.414	282.462[a]	167.637	606.738[a]	250.939	636.731[a]	306.541
B	0.1% Atonik	26.707[ab]	30.358	157.696[a]	142.645	262.022[a]	205.485	594.685[a]	350.311	656.700[a]	362.343
C	0.2% Atonik	21.079[b]	27.861	154.476[a]	166.610	254.805[a]	213.368	576.037[a]	388.422	606.859[a]	415.879
D	0.5% Atonik	0	0	0	0	79.620b*	113.488	255.575[b]*	183.441	384.024[b]*	290.327

Note: Measured in µm, x4 magnification. Means with different subscripts have a significant difference ($p < 0.05$), means with * differ ($p < 0.01$)

Pollen germinated under 10 °C yielded rates with a statistically insignificant differences between variant A which had a rate of 49.97% (Table 6), B with 44.46% and C with 36.28% rate. Variant D did not germinate at all.

Under 15 °C pollen germinated at statistically indifferent rates, variant A at 65.72%, variant B at 63.24% and variant C at 56.68%. Variant D again did not germinate even after 4 hours.

At 18 °C best pollen germination rate was 66.21% of variant A, compared to variant B 61.65% and variant C 57.82% no statistically significant difference was found. Worst pollen germination was found in variant D at 13.84%, the rate only very slightly grew from the rate at 2 hours of cultivation. Difference between variant D and the other variants was statistically significant.

The germination rate of pollen under 26 °C was again, with no statistically significant differences between the three variants, A at 69.79%, B at 62.87% and C at 54.18%. Variant D had worst rate at 40.71% with a statistically significant difference when compared to the other variants.

At 30 °C pollen germination rates were the best between the temperatures (Figure 11). Variant A germinated at 80.11%, variant B at 74.66% and variant C at 55.45%. Lowest germination rate was found in variant D with 55.45%, it was statistically significantly different then variants A and B.

Table 6: Pollen germination percentage after 4 hours of cultivation.

Treatment		Temperature									
		10 °C		15 °C		18 °C		26 °C		30 °C	
		Mean $\bar{x}$	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*	*Mean $\bar{x}$*	*SD*
A	Control	49.97^{a}%	7.45	65.72^{a}%	8.82	66.21^{a}%	10.84	69.79^{a}%	7.32	80.11^{a}%	8.41
B	0.1% Atonik	44.46^{a}%	10.68	63.24^{a}%	7.36	61.65^{a}%	7.57	62.87^{a}%	21.15	74.66^{a}%	4.51
C	0.2% Atonik	36.28^{a}%	10.82	56.68^{a}%	9.10	57.82^{a}%	7.31	54.18^{a}%	20.94	69.95^{ab}%	6.65
D	0.5% Atonik	0	0	0	0	13.84^{b}*%	10.58	40.71^{b}*%	3.84	55.45^{b}*%	15.76

Note: Means with different subscripts have a significant difference ($p < 0.05$), means with * differ ($p < 0.01$).

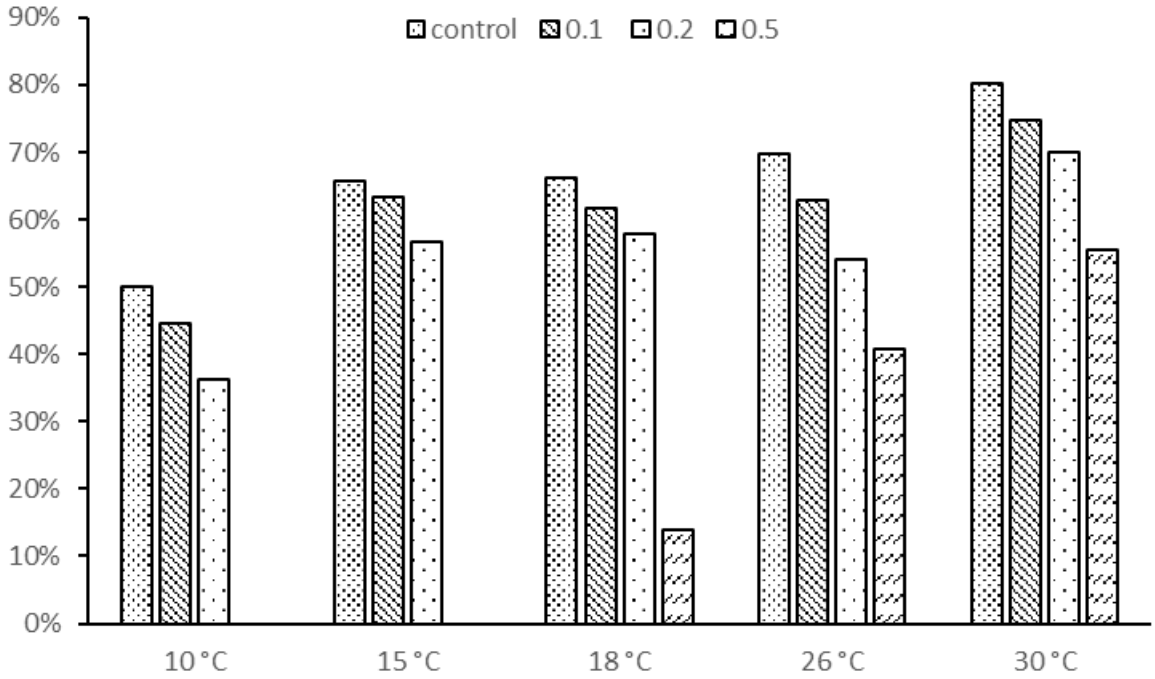

Figure 11: Pollen germination after 4 hours of cultivation.

5.4 Pollen tube growth rate

Exponential trendlines of pollen tube lengths under 10 °C (Figure 12) show close growth rates between variants A, B and C with possible differences emerging after 6 hours of cultivation. At 15 °C (Figure 13) the rates are nearly identical, showing no significant differences. Under 18 °C (Figure 14) variants A, B and C experience exponential while variant D starts a linear growth only after the first hour has passed. At 26 °C (Figure 15) and 30 °C (Figure 16) the three variants A, B and C experience similar exponential trendline tube growth while variant D in both temperatures trails significantly behind them.

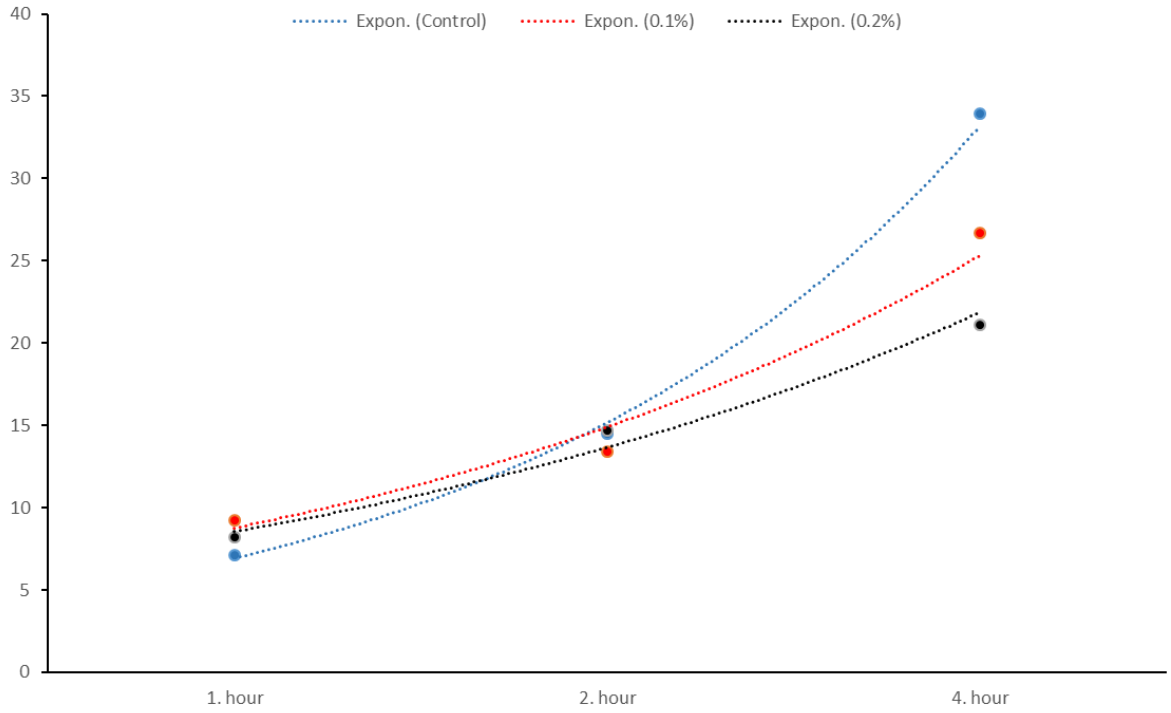

Figure 12: Pollen tube lenght in µm x4, cultivated in 10 °C with exponential trendlines.

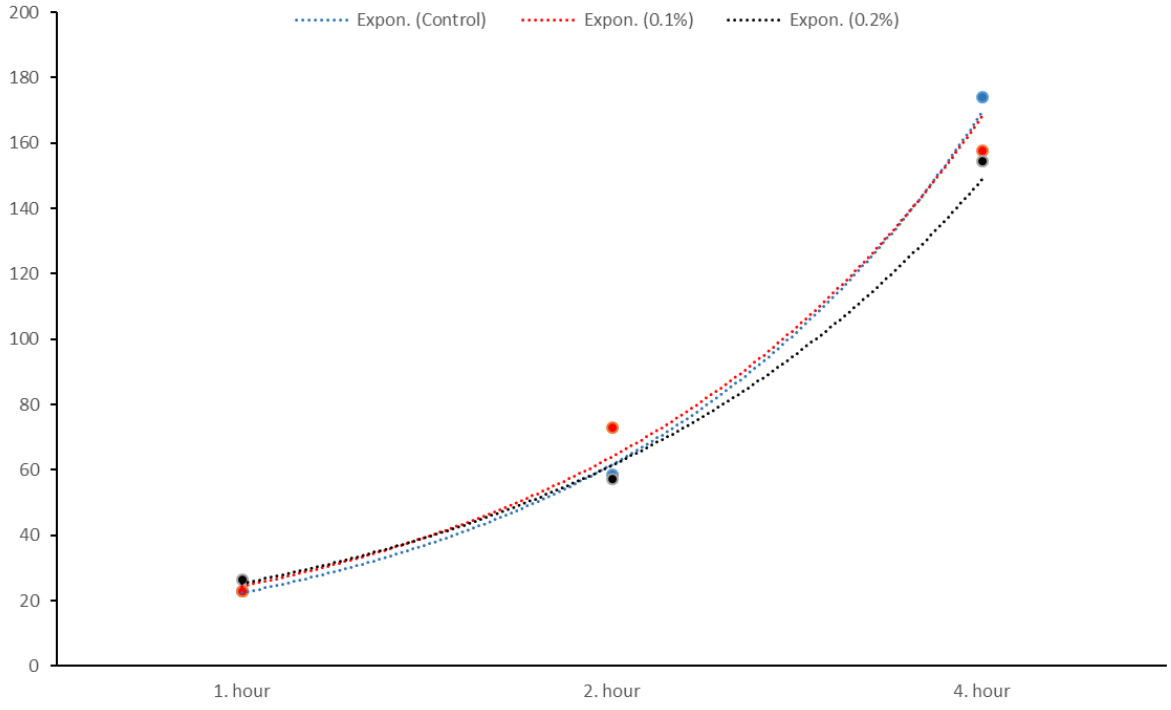

Figure 13: Pollen tube lenght in µm x4, cultivated in 15 °C with exponential trendlines.

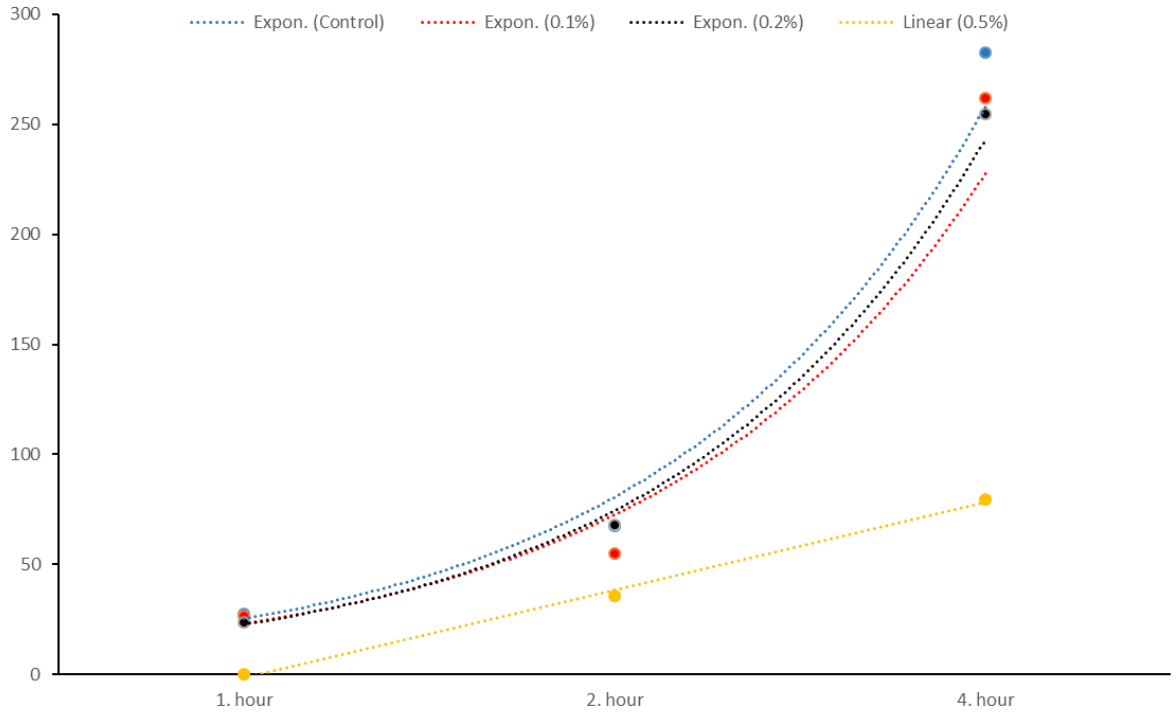

Figure 14: Pollen tube lenght in µm x4, cultivated in 18 °C with exponential trendlines and a linear line.

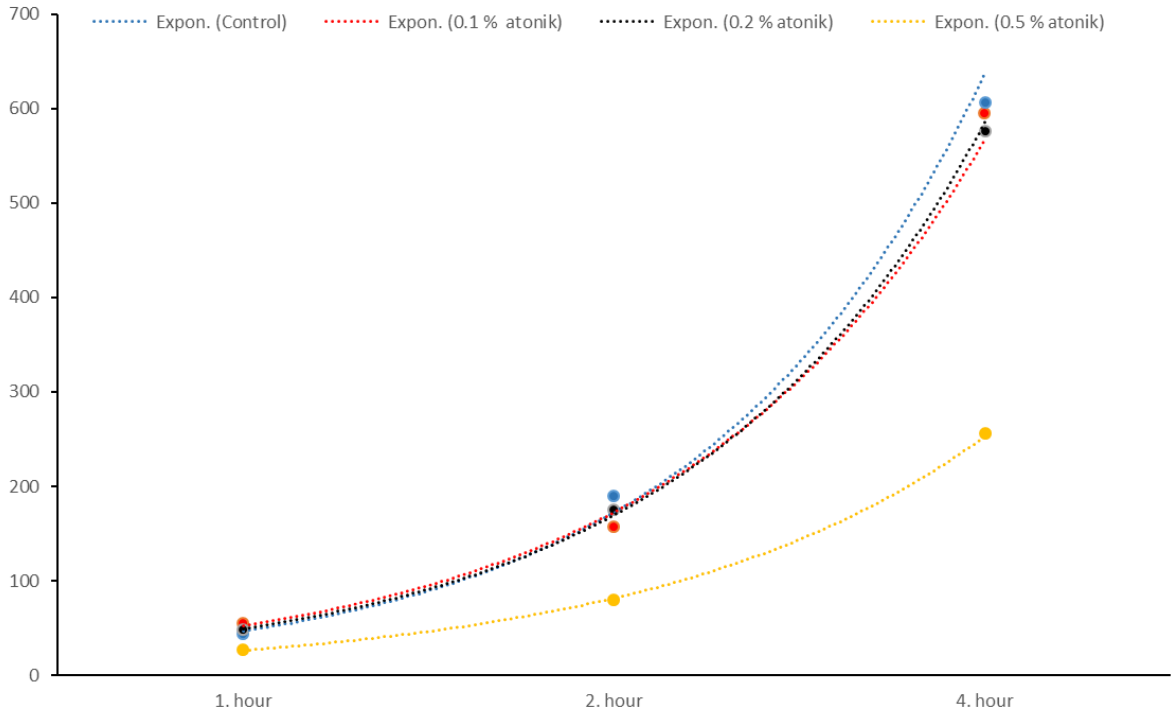

Figure 15: Pollen tube lenght in µm x4, cultivated in 26 °C with exponential trendlines.

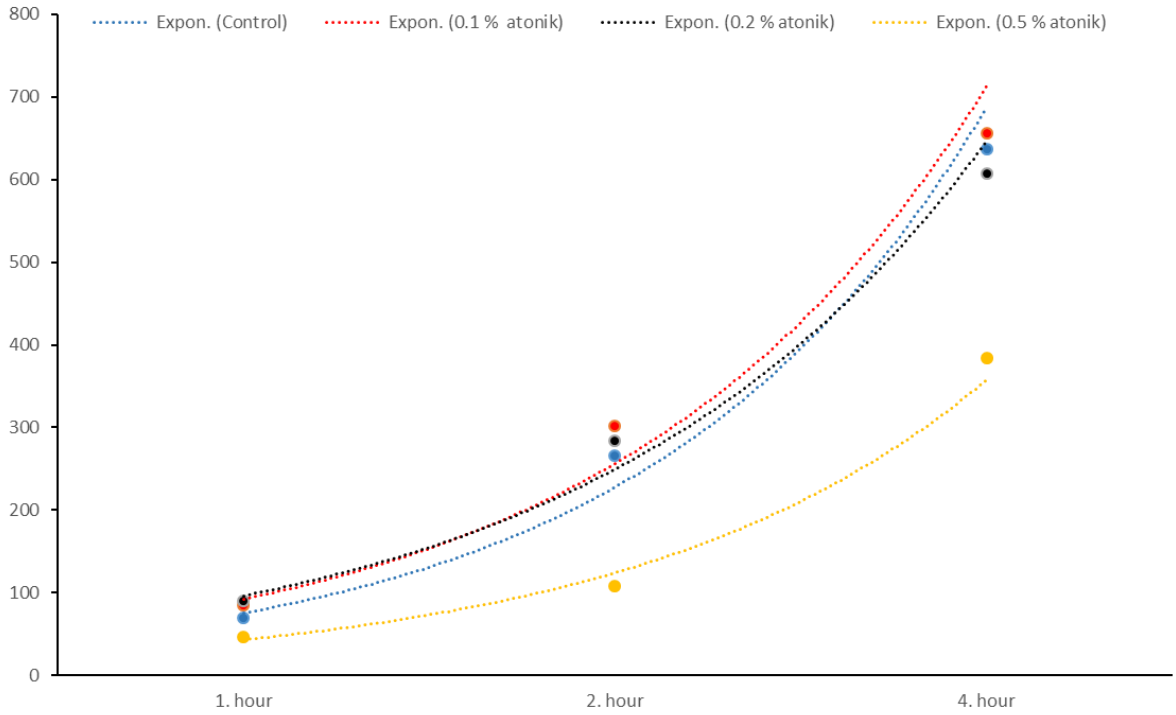

Figure 16: Pollen tube lenght in µm x4, cultivated in 30 °C with exponential trendlines.

6 Discussion

Pollen germination and tube growth are important factors in plant productivity, as marketable products of most agricultural plants are the seeds. Atonik is a biostimulant product that has been shown to positively affect the plant physiological processes (Przybysz et al. 2014). This work deals with the direct effect of Atonik in 0.1%, 0.2% and 0.5% on pollen germination and tube growth of tobacco (*Nicotiana tabacum* cv. Samsun) *in vitro*. It is the only research concerning the effect of these concentrations.

The methodology is done according to standard practices of research on pollen in the Institute of experimental botany of the czech academy of sciences. While the flowers were left in a fume box to dehisce at room temperature in a similar manner, in study by Sorkheh et. al (2011) left the anthers of almond plants to dehisce with silica pallets for 2 to 3 days at room temperature in a glass jars, after which the collected and stored with silica pallets in airtight glass jars. In both studies the pollen grains were kept at -20 °C. Alexander (2019) used newly opened flowers with pollen that was visibly dehisced on each day of testing. The approach of using newly collected pollen grains was also used by Kakani et al. (2005). The assessment of germinated pollen differed, in our study all pollen grains with a visible protruding pollen tip at least 4 µm was considered as germinated, in other research only pollen grains with a tube longer or equal to the diameter of the pollen grain was considered as germinated (Sorkheh et al. 2011). In this research pollen germination and tube length were measured after 1 hour, 2 hours and 4 hours of cultivation, this short time window made it necessary to calculate pollen with small protruding tips as germinated, in cases with a larger time window a stricter criterion for germination classification could be more suitable.

It has been shown that storage and temperature stress could affect pollen viability and vigour. Stressed pollen took longer to germinate reaching maximum germination after 6 hours unlike fresh pollen which germinated over 80% within the first hour. However, reports showed it did not affect overall germinability, such as in *Malus domestica* pollen which was stored for 1 year with no loss of *in vitro* germinability (Shivanna et al. 1991). Our results show a 56.65% germination within the first hour in the control variant under 26 °C with a statistically insignificant differences in the germination of pollen grains with 0.1% Atonik at 56.71% and

0.2% Atonik at 53.28%. This suggests the storage of pollen did not have a significant effect on the germination and neither did Atonik in 0.1% and 0.2% concentrations. Pollen germinated at similar rates between these three treatments under the different temperatures of 10 °C, 15 °C, 18 °C, 26 °C and 30 °C. The colder cultivation temperature of 10 °C did stress all three variants drastically slowing their germination. After two hours pollen grains with 0.2% Atonik under 10 °C germinated at 31.91 a statistically significant worst percentage than control variant, however no such effect was found under the other temperatures. After four hours have passed, this inhibitory effect of 0.2% was not found, and pollen of control, 0.1% and 0.2% Atonik germinated at similar rates. This suggest that 0.2% Atonik could have a slight inhibitory effect on germination under extreme temperature stress however under the tested temperatures 0.1% and 0.2% Atonik does not cause a positive or negative effect on the pollen germination.

Ylstra et al. (1995) studied pollen germination and tube growth of tobacco pollen *in vitro* under different concentrations of hormones and found a stimulatory effect of flavanols when adding it in 0.3 µM to 1 µM concentrations, mammalian steroids also resulted in an increased germination frequency in 2 to 20 µM concentrations. In contrast sterols and plant hormones did not stimulate pollen development, having no enhancing effect on pollen germination. In a paper Viti et al. (1989) researched the effect of growth regulators on pollen germination of olive plants. Biostimulant Siapton 10L was applied *in vivo* and *in vitro* at concentrations of 0.05 % and 0.1 %. Gemination was determined at 24 °C. It was found that Siapton did not have a favourable effect on germination ability *in vitro* however it did on *in vivo* pollen tube elongation. This agrees with our results which found no positive effect of 0.1% and 0.2% Atonik on *in vitro* pollen germination.

Pollen with 0.5% Atonik treatment had a clear inhibitory effect on pollen germination. Under 10 °C and 15 °C cultivation conditions the pollen grains did not germinate even after four hours have passed. At the temperature of 18 °C pollen started to germinate after the second hour at 12.94%, after four hours it only reached 13.84. Under At 26 °C and 30 °C the germination rate was better at 15.10% and 25.64% respectively in the first hour and reaching 40.14% and 55.45%, these rates however were still statistically significantly lower than the control variant. The pollen tube elongation was also affected, tube lengths reached only half

the lengths of the control variant with statistically significant differences. This inhibitory effect could be caused by Atonik in 0.5% concentration altering the ion gradient necessary for germination and tube growth, in the lower temperatures an additional temperature stress lead to the pollen losing its ability to germinate. According to An et al. (2018) 5-Aminolevunic acid inhibits pollen tube growth by causing a Ca^{2+} efflux. 0.5% Atonik could inhibit pollen tube growth in a similar manner.

Pollen tube length in pollen grains treated with 0.1% Atonik showed longer tubes at 9.245 µm under 10 °C after one hour of cultivation compared to the control variant at 7.087 µm. Under 30 °C 0.2 % has also shown a positive effect at 90.332 µm compared to control at 69.882 µm. However, the measurements after two hours showed this effect did not persist with pollen tube lengths of 0.1% and 0.2% Atonik having statistically insignificant differences to control. After 4 hours 0.2% Atonik under 10 °C had shorter tubes compared to control, in the other tested temperatures no statistically significant differences in pollen tube length were found in 0.1% and 0.2% Atonik compared to the control variant. These results show that Atonik in 0.1% and 0.2% concentrations does not have a consistent positive effect on pollen growth. Further measurements after six hours of growth and *in vivo* testing of pollen elongation could yield clearer results on Atonik effect, as Viti et al. (1989) results showed a positive effect of Siapton 10L biostimulant on olive pollen growth *in vivo*. In a study Filiti et al. (1986) sprayed Siapton and SA-100/G3 (aminoacids and animal by-product peptides) was sprayed at 0.2% and 0.3% at flowers of fruit trees, pollen was then removed and assessed. Both biostimulants were found to induce a moderate increase in plum pollen germination and an increase in pollen tube growth in all tested fruit trees.

The higher yields of plants after treatment with Atonik could be the result of an increased photosynthetic efficiency which in turn increases the production of photosynthates resulting in a translocation of organic materials from source to sink (Djanaguiraman et al. 2005b). As pollen grains do not possesses chloroplasts such a positive effect of Atonik would not be found during *in vitro* pollen development.

7 Conclusion

The effect of Atonik on the progamic phase of the male gametophyte was evaluated. The following results were concluded:

- Atonik at 0.1%, 0.2% and 0.5% concentrations in the cultivation medium did not have positive effect on pollen germination. No significant difference between pollen germination in medium with 0.1%, 0.2% Atonik and control variant was observed. 0.5 % Atonik had a significant inhibitory effect on pollen germination especially under colder temperatures.

- Atonik at 0.1%, 0.2 % and 0.5% concentrations in the cultivation medium did not have positive effect on pollen tube elongation. No significant differences between pollen tube elongation in medium with 0.1%, 0.2% Atonik and control variant was observed. 0.5 % Atonik had a significant inhibitory effect on pollen tube growth.

- Obtained results cannot necessarily be generalized to *in vivo* application and to other plant species.

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