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The Responses of Pollen Tubes to Spermidine Treatments in *Actinidia deliciosa*

Actinidia deliciosa'da Polen Tüplerinin Spermidin Uygulamalarına Verdiği Yanıtlar

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Abstract: In this study, the responses of pollen tubes to spermidine treatments (10 µM, 25 µM, 50 µM, 100 µM, 250 µM, or 500 µM) were investigated in *Actinidia deliciosa*, by focusing on pollen germination rate, pollen tube length, organizations of actin filaments, concentrations of Ca^{+2}, pH, reactive oxygen species and distributions of callose and cellulose. According to findings, the only positive effect was detected after 10 µM spermidine treatment while the most negative acute effect was detected after 500 µM spermidine treatment and, further experiments were done in these groups. 10 µM spermidine increased the pollen tube length by changing the concentration of apex localized reactive oxygen species. 500 µM spermidine decreased the pollen tube length by changing the apex localized Ca^{+2}, pH, and reactive oxygen species concentration. Findings would contribute to the understanding of the effects of polyamines on pollen tubes.

Keywords: Actin cytoskeleton, cell wall, pollen tube, polyamine, spermidine

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Öz: Bu çalışmada *Actinidia deliciosa*'da polen tüplerinin spermidin uygulamalarına (10 µM, 25 µM, 50 µM, 100 µM, 250 µM veya 500 µM) verdiği yanıtlar, polen çimlenme oranı, polen tüp uzunluğu, aktin filament organizasyonu, Ca^{+2}, pH, reaktif oksijen türlerinin konsantrasyonu ve kalloz ve selüloz dağılımına odaklanarak incelenmiştir. Bulgulara göre tek olumlu etki 10 µM spermidine uygulamasından sonra tespit edilirken, en olumsuz akut etki 500 µM spermidine uygulamasından sonra tespit edilmiş ve ileri deneyler bu gruplarda yapılmıştır. 10 µM spermidin, apekste lokalize olan reaktif oksijen türlerinin konsantrasyonunu değiştirerek polen tüpü uzunluğunu arttırmıştır. 500 µM spermidin ise apekste lokalize olan Ca^{+2}, pH ve reaktif oksijen türlerinin konsantrasyonunu değiştirerek polen tüpü uzunluğunu azaltmıştır. Bulguların poliaminlerin polen tüpleri üzerindeki etkilerinin anlaşılmasına katkıda bulunabileceği düşünülmektedir.

Anahtar Kelimeler: Aktin hücre iskeleti, hücre çeperi, polen tüpü, poliamin, spermidin

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INTRODUCTION

Kiwifruit (*Actinidia deliciosa*) is a plant with a high economic value, spreading around the world and Türkiye (Dutta et al., 2023). Its fruits are often used as raw materials in the pharmaceutical, cosmetic, food and beverage industries. It has become a more popular fruit in recent years due to its low-calorie content, and positive results in protection from cancer. About this, studies to increase fruit production have also gained momentum (Garg et al., 2023). Kiwifruit is a dioecious plant. Stamens of female flowers are infertile. However, the stamens of male flowers produce a great number of fertile pollen grains. For fruit formation, pollen grains produced in male flowers must be transferred to the stigma of female flowers. As in many plants, fruit formation in kiwifruit depends mainly on the effort of pollination and fertilization. The main elements of pollination and fertilization are pollen tubes (PTs) (Güçlü et al., 2020).

PTs lengthen by transporting the secretory vesicles including the cell wall materials to the PT apex via actin filaments. Many factors such as calcium (Ca^{+2}), reactive oxygen species (ROS), and pH presumably generates the fitting circumstance for secretory vesicles to proceed toward a particular point on the apical plasma membrane (Zhao et al., 2023). Thereupon, ongoing remodeling of the cell wall balances the PT growth. As it turns out many intracellular factors within the PTs play coordinated roles in the elongation. For instance, there is a crosstalk between Ca^{+2} and ROS and this crosstalk regulates the actin filament organization which is also regulated by pH concentration within the PTs. Alterations in actin filament organization cause the changes in the distribution of main PT cell wall materials named callose and cellulose (Kapoor and Geitmann, 2023). Although the PT can be divided into different parts according to different characters, the most important part of the PT is undoubtedly the apex region. Because this region contains regulative items such as Ca^{+2}, ROS, and pH, which are requisite for the elongation of the PT, at a suitable concentration at the PT apex (Tang et al., 2023). As well, actin filaments in this region are very short and active, so they can briskly restyle to current growing ambiences. Although cellulose is present in the PT wall in this region, callose is usually absent. All these characters are specially arranged in this region for the elongation of the PT (Zhang et al., 2023).

Any alteration of the multicomponent mechanism alters pollen germination (PG) and PT elongation. Some plant growth regulators affect PG and PT elongation by influencing these factors (Jinming et al., 2023). Polyamines (PAs) lead these plant growth regulators and it has especially been known some types of PA such as putrescine and spermine are effective on PG and PT elongation (Aloisi et al., 2016; Benko et al., 2020). Although it has been reported that exogenic spermidine (Spd) treatments affect PT elongation in various species such as *Malus domestica* (Del Duca et al., 1997), *Lycopersicon esculentum* (Song et al., 1999), *Prunus mume* (Wolukau et al., 2004), *Prunus dulcis* (Sorkheh et al., 2011) and *Camellia sinensis* (Çetinbaş-Genç et al., 2020), there is limited information on how Spd affects the factors that work coordinately in PT elongation. However, it is of great importance to determine the concentrations of Spd that increase PG and PT elongation and to determine how this increase modulates the processes that play a role in PT elongation. Especially in plants such as kiwifruit, where artificial pollination is quite common practice, it is very likely that the use of PA types and appropriate concentrations that increase PG and PT elongation in artificial pollination processes will increase product yield. However, when all studies on PAs and PT elongation were examined, it was seen that there was no study examining the effects of different Spd concentrations on PT elongation in kiwifruit.

This study aims to examine the intracellular effect of Spd on kiwifruit PTs by focusing on PG rate, PT length, actin filaments, Ca^{+2}, pH, ROS, callose, and cellulose, and also evaluate the cytological responses that occur in PTs after Spd treatments. It is thought that the findings will contribute to both the artificial pollination processes of kiwifruit and the understanding of the effects of PAs on PTs.

MATERIAL AND METHOD

Pollen grains were collected from male flowers of *Actinidia deliciosa*'s Matua cultivar in a kiwifruit orchard located in Akçakoca/Düzce, in 2022 summer. Pollen grains dehyrated in silica gel for overnight and stored at -20 °C until the use. were germinated for 3 hours in Brewbaker and Kwack's medium with 12% sucrose. 0 μM, 10 μM, 25 μM, 50 μM, 100 μM, 250 μM, or 500 μM Spd was added to germination

medium (Vogler et al., 2014). PG rates and PT lengths were calculated by examining at least 150 pollen grains and PTs, respectively. Cumulative stress response index (CSRI) values were calculated according to the formula of Dai et al. (1994), using the results of PG and PT lengths to get cumulative results. Further experiments were done in the groups showing the most negative and positive effects detected by CSRI values. PTs were labeled with Alexa 488-phalloidin after the fixation process as described by Lovy-Wheeler et al. (2005) and were examined with a fluorescence microscope at 488-515 nm. Actin filament anisotropy levels at the area that covers 100 μm^2 of the apex were measured using the "Fibril Tool" plugin of ImageJ software, in at least 20 PTs (Boudaoud et al., 2014). PTs were labeled with 5 µM Chlortetracycline hydrochloride (CTC) for Ca^{+2}, 5 µM 2',7'-bis-(2-Carboxyethyl)-5-(and-6)-carboxyfluorescein acetoxymethyl ester (BCECF-AM) for pH (Serrazina et al., 2014) and 20 µM 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) for ROS (Malho et al., 2000). Ca^{+2}, pH, and ROS distributions were examined with a fluorescence microscope at 515-536 nm, 440-490 nm, and 500-535 nm, respectively. Fluorescence intensities of probes at the area that covers 100 μm^2 of the apex were measured using the ImageJ software, in at least 20 PTs. PTs were stained with 0,1% Aniline blue for callose (Wang et al., 2003) and 1% Calcofluor white for cellulose (Derksen et al., 2002). Callose and cellulose accumulations were examined by a fluorescence microscope at 455-495 nm and 365-432 nm, respectively. Fluorescence intensities of stains at the area that covers 100 μm^2 of the apex using the 'Rectangle Selection' option of ImageJ software, in at least 20 PTs. All measurements were made in 3 repetitions. All measurement and calculation results were statistically compared by the ONE-WAY ANOVA test using the SPSS program (16.0). In the graphs, distinct letters point out the statistically significant differences ($P < 0.05$) and error bars indicate the standard deviations.

RESULTS

Foremost, PG rates and PT lengths were determined to gain basic information about the effect of Spd. Results showed that Spd treatments did not generate a significant change in PG rates when compared to the control (Figure 1a). PT lengths were significantly increased by 13.85% after 10 µM Spd treatment. However, PT lengths were significantly decreased by 24.60% after 25 µM, by 22.97% after 50 µM, by 24.62% after 100 µM, by 21.65% after 250 µM and by 25.92% after 500 µM Spd treatment, compared to the control (Figure 1b).

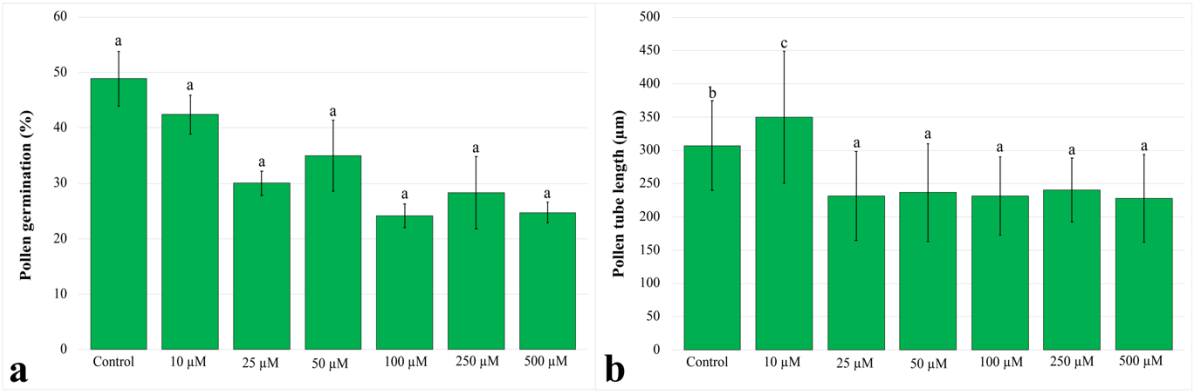

Figure 1. Effect of different Spd concentrations on PG and PT length. a. PG rates, b. PT lengths.
Şekil 1. Farklı Spd konsantrasyonlarının polen çimlenmesi ve polen tüp uzunluğuna etkisi. a. Polen çimlenme oranları, b. Polen tüp uzunlukları.

To determine the concentrations with the most negative and positive effects on PT, the CSRI values were calculated. According to CSRI values, the only positive effect was detected after 10 µM Spd. Although all other groups showed negative effects, the most negative acute effect was detected after 500 µM Spd (Table 1). According to CSRI results, further experiments were done in control, 10 µM, and 500 µM Spd groups.

Table 1. CSRI values.
Çizelge 1. CSRI değerleri.

	10 µM	25 µM	50 µM	100 µM	250 µM	500 µM
CSRI	0.59	-63.15	-51.41	-75.22	-63.80	-75.33
Evaluation key	Negative < -46.80 < Medium < -18.27 < Positive < 10.25					

In order to reveal Spd-induced changes in intracellular features at the apex, actin cytoskeleton organization, Ca^{+2}, pH, and ROS distributions were observed at the area that covers 100 µm² of the apex. The results showed that Spd treatments did not generate a significant alteration in the distribution of actin filaments at the apex (Figure 2a). Ca^{+2} and pH concentrations did not change after 10 µM Spd treatment. However, after 500 µM Spd treatment, Ca^{+2} and pH concentration increased statistically by 76.35% and 185.54%, respectively (Figure 2b,c). Concentrations of ROS decreased statistically by 16.66% after 10 µM Spd while increasing statistically by 9.42% after 500 µM Spd treatment (Figure 2d).

In order to reveal Spd-induced changes in cell wall properties, callose and cellulose accumulations were observed at the area that covers 100 µm² of the apex. The callose accumulation increased statistically by 58.30% after 10 µM and by 181.30% after 500 µM Spd treatment (Figure 2e). However, there was no statistical difference in cellulose deposition after Spd treatment (Figure 2f).

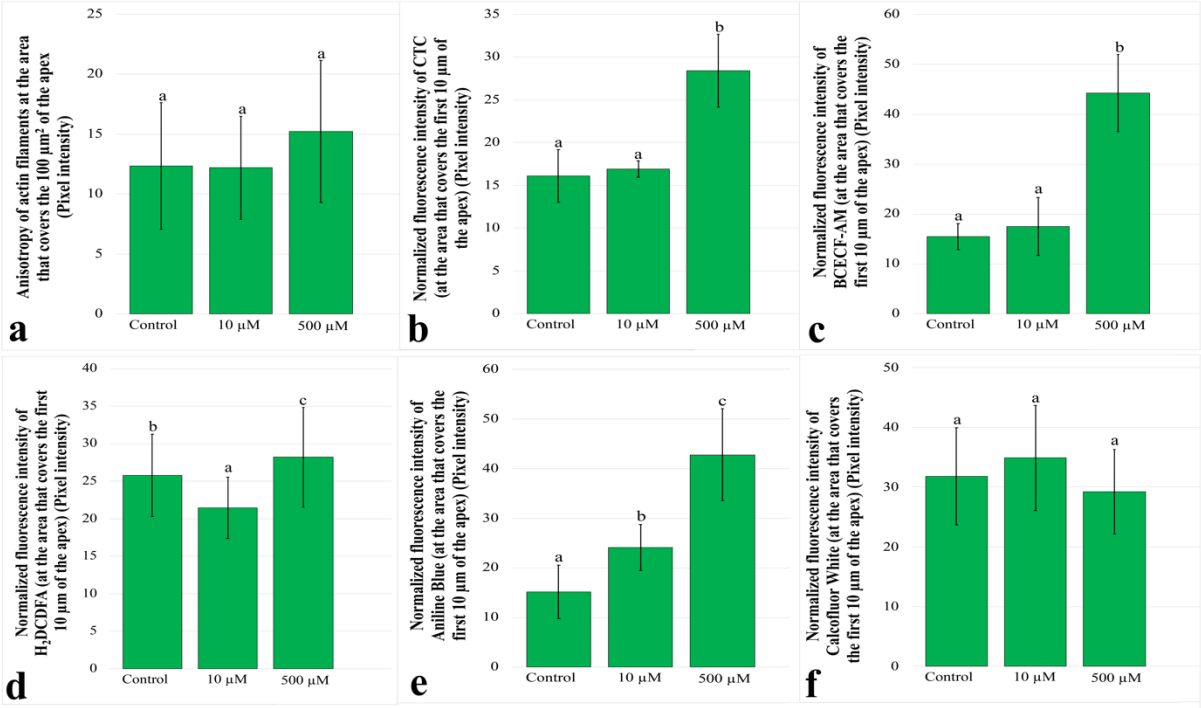

Figure 2. Spd induced changes in intracellular features and cell wall properties at the area that covers 100 µm² of the apex. a. Anisotropy values of actin filaments. b. Ca^{+2} distributions, c. pH distributions, d. ROS distribution, e. Callose accumulation, f. Cellulose accumulation.
Şekil 2. Apeksin 100 µm²'sini kaplayan alanda hücre içi ve hücre çeperi özelliklerinde Spd kaynaklı değişiklikler. A. Aktin filamentlerin anizotropi değerleri, b. Ca^{+2} dağılımı, c. pH dağılımı, d. ROS dağılımı, e. Kalloz birikimi, f. Selüloz birikimi.

DISCUSSION

Researchers have reported that appropriate doses of putrescine and spermine increase PG and PT elongation in many different species (Sorkheh et al., 2011). However, little is known about the role of Spd, another important PA type, in PG and PT elongation (Wu et al., 2010). For example, 50 µM Spd has been reported to increase PG and PT elongation in both *Lycopersicum esculantum* (Song et al., 1999) and *Nicotiana tabacum* (Benko et al., 2020). Additionally, it was determined that 25 µm and 250 µm Spd caused the formation of longer PTs in *Prunus mume* and *Prunus dulcis* (Wolukau et al., 2004; Sorkheh et al. 2011). As can be understood, almost most of these studies reported how Spd increased PG and PT elongation. However, more studies are needed to explain how Spd, which has a high ability to interact with different molecules and to regulate many different pathways within the cell, affects the molecules and processes that enable PT to elongate (Aloisi et al., 2022). In this study, it was determined that different Spd doses did not cause a significant change on PG, but 10 µM Spd caused the formation of long PTs. However, all other concentrations decreased the PT length. The fact that 50 µM Spd in *Lycopersicum esculantum* and *Nicotiana tabacum* or; 250 µM Spd in *Prunus mume* and *Prunus dulcis* increased PT length, but these concentrations reduced PT length in *Actidinia deliciosa*, supports the notion that the effects of PAs may differ between species (Zhang et al., 2023).

In this study, it has been tried to determine the concentrations that show the most positive and negative acute effects on PG and PT elongation. Although Spd doses did not have an effect on PG, since they created significant changes on PTs. For this reason, results of PG and PT length were evaluated cumulatively as stated in the literature (Dai et al., 1994). Similarly, researchers evaluated PG rate and PT lengths cumulatively in their studies on *Olea europaea* and *Prunus dulcis* (Koubouris et al., 2009; Sorkheh et al., 2018). As a result of the cumulative evaluation, the concentrations showing the best and worst effects were determined to be 10 µM and 500 µM, respectively, and further experiments were carried out only in these groups.

It is known that PAs regulate PT elongation by modulating the activities of the actin cytoskeleton (Aloisi et al., 2016). It has been revealed that especially the actin filaments in the apex must be extra active to adapt to changing growth conditions and the actin filaments in this region are much more important for PT elongation than other regions (Cai et al., 2015). So much so that many researchers have revealed that an increase in actin filament anisotropy at the apex causes the formation of short PTs, while a decrease in anisotropy causes the formation of long PTs (Del Duca et al., 2013; Aloisi et al., 2016). Nonetheless, in this study, no significant change in actin filament anisotropy at the apex was detected after any Spd concentration. For this reason, it can be concluded that the acute changes detected in growth parameters after 10 µM and 500 µM Spd treatments are not actin-related.

In PTs that show proper elongation, the apex of the PT exhibits high Ca^{+2}, pH and ROS concentration (Wu et al., 2010). Many researchers have stated that deterioration in Ca^{+2} concentration at the apex is associated with the inhibition of PT elongation (Nie et al., 2023). Based on this information, the high increase observed in Ca^{+2} and pH concentration at the PT apex after 500 µM Spd treatment explains the sharp decrease in PT length in this group. Similarly, it has been reported that 100 µM spermine causes short PT formation in *Pyrus communis* by causing sharp changes in Ca^{+2} and pH concentration at the PT apex (Aloisi et al., 2016). Researchers have stated that apex localized ROS play a role in the remodeling of actin filaments by modulating the apex localized Ca^{+2} concentration (Potocky et al., 2012; Jinming et al., 2023). In this study, the changes observed in ROS deposition at the PT apex following the 10 µM Spd treatment were not reflected in the Ca^{+2} and pH levels at the apex and, did not show any change in actin organization. These findings suggest that 10 µm Spd modulates PT elongation only by making changes in ROS accumulation. Also similar to these findings, Benko et al. (2020) stated that 10 µM Spd application in *Nicotiana tabacum* increased PT lengths and reduced ROS levels in PTs. On the other hand, it was determined that after 500 µM Spd application, ROS accumulation modulated the Ca^{+2} and pH concentration, but this modulation was not reflected in the actin filament organization. Therefore, it was thought that 500 µM Spd modulated PT elongation in coordination with Ca^{+2}, pH and ROS. It has also been reported that PT lengths decreased and ROS levels increased after 1 mM Spd application in

Arabidopsis thaliana (Wu et al., 2010). Also, researchers have stated that PA-induced changes in apex localized Ca^{+2}, pH and ROS concentrations can directly affect the structure of the PT cell wall (Parrotta et al., 2022). In this study, there was no change in the cellulose distribution on apex after both 10 µM and 500 µM Spd application. However, callose deposition at the apex increased following the treatment with both concentrations. Similarly, it has been reported that rich callose deposition was observed at the apex of the PT in *Pyrus communis* pollen grains exposed to 100 µM spermine (Aloisi et al. 2017).

CONCLUSION

10 µM Spd treatment increases PT length by changing the apex localized ROS concentration. 500 µM Spd treatment decreases PT length by changing the apex localized Ca^{+2}, pH, and ROS concentration. Results show how exogenic Spd treatment impinges the processes and molecules involved in PT elongation in the most prominent examples. Obtained findings would contribute to both the artificial pollination processes of kiwifruit and may conduce to the understanding of the cellular activity mechanism of PAs in both PTs and other structures extending from the tip.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

DECLARATION OF AUTHOR CONTRIBUTION

All authors contributed equally.

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