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Saliciylic Acid is also Effective Along with Abscisic Acid and Gibberellic Acid in the Orchid Post-Pollination Process

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ABSTRACT

The aim of this study is to determine the endogenous hormone activities of abscisic acid (ABA), Jasmonic acid (JA) and Salicylic acid (SA) in compatible (Intraspecific = ISP) and incompatible pollination (Intergeneric = IGP) in stigmatic ovaries during orchid post-pollination process. ISP pollination experiments were conducted among Himantoglossum robertianum flowers. In IGP experiments, pollination was carried out between the flowers of *Himantoglossum robertianum* and *Orchis italica*. For this, polynariums taken from *O. italica* have been applied with needles to the flower stigmas of *H. robertianum*. Ovaries with stigma from both pollination types were taken separately for each day for a total of 10 days and quantitative endogenous hormone analyzes have been performed by LC-MS/MS. After all, SA hormone has been detected for the first time in both ISP and IGP, but only ISP values were significant. The statistical analysis revealed that while SA is significant in only ISP, ABA values were found to be highly significant in both ISP and IGP. ISP9 also showed high significance. Additionally, ABA values were generally higher than SA values, especially in IGP experiments. The results also emphasized the importance of ovarian endogenous hormone activities for the first time. JA was not detected in the analysis.

Salisilik Asit Orkidelerin Tozlaşma Sonrası Sürecinde Absisik Asit ve Gibberellik Asit ile Birlikte Etkilidir

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ÖZ

Bu çalışmanın amacı orkide stigmalı ovaryumlarında uyumlu (Intraspesifik = ISP) ve uyumsuz tozlaşmada (Intergeneric = IGP) absisik asit (ABA), Jasmonik asit (JA) ve Salisilik asitin (SA) endojen hormon aktivitelerini belirlemektir. ISP tozlaşma deneyleri *Himantoglossum robertianum* çiçekleri arasında yapılmıştır. IGP deneylerinde tozlaşma, *Himantoglossum robertianum* ve *Orchis italica* çiçekleri arasında gerçekleştirildi. Bunun için *O. italica*'dan alınan polinariumlar, *H. robertianum*'un çiçek stigmalarına özel iğnelerle uygulanmıştır. Her iki tozlaşma tipine ait stigmalı ovaryumlar toplam 10 gün boyunca her gün için ayrı ayrı alınmış ve LC-MS/MS ile kantitatif endojen hormon analizleri yapılmıştır. Sonuçta SA hormonu ilk kez hem ISP'de hem de IGP'de tespit edildi ancak yalnızca ISP değerleri anlamlı çıkmıştır. Yapılan istatistiksel analizde SA'nın sadece ISP'de anlamlı olduğu, ABA değerlerinin ise hem ISP'de hem de IGP'de oldukça anlamlı olduğu görüldü. ISP9 da yüksek önem gösterdi. Ayrıca ABA değerleri, özellikle IGP deneylerinde genellikle SA değerlerinden yüksekti. Sonuçlar aynı zamanda ilk kez ovaryum endojen hormon aktivitelerinin önemini de vurguladı. Analizlerde JA tespit edilmemiştir.

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1. Introduction

Considered being the largest family of plants, Orchidaceae consists of 25,000–35,000 species (Attri et al., 2020). Orchids have been the focus of attention of many researchers and animals with their labellums, which varied in shape and color, and their patterned flower structures consisting of 3 petals and 3 sepals. Orchids are considered as unusual among flowering plants (Withner et al., 1974). Food deceptive and rewarding flowers, which are seen as pollination strategies, or attractive flower structures that secrete sex deceptive and chemical pheromones attract the attention of many pollinator agents (Lanzino et al., 2023). After such reciprocal pollination events with pollinator insects, the complex events in flower structures and metabolisms are entitled as "post pollination phenomena" and the findings on this subject are outlined below:

The first action that triggers hormonal events in post-pollination is the penetration of auxin in the pollen into the stigma (Arditti and Flick, 1976; Strauss and Arditti, 1984). Auxin from pollen diffuses through the columna and stimulates ethylene production, which triggers perianth senescence (Clifford and Owens, 1988). Orchid flowers are very sensitive to ethylene (Dijkman and Burg, 1970). Ethylene is also released in the flower after the polynarium leaves the flower by a pollinator insect (Arditti et al., 1973; Strauss and Arditti, 1984) and with the activity of auxin, the amount of ethylene increases even more in the flower (Strauss and Arditti, 1984). Therefore, in the post-pollination processes, the rostellum and stigmatic parts of the flower are the controlling regions of many events. Rostellum is a median stigma region that initiates and regulates a series of post-pollination phenomena (Arditti and Flick 1974; Arditti 1979a) by producing ethylene, and the flower segments display localized effects only within its area in this process. For instance; Absisic acid (ABA) and Naphtalen acetic acid (NAA), ethylene and partially Gibberellic acid (GA) in the lateral petals, sepals and labellum, yellowing and hooding in the dorsal sepal, senescens and anthocyanin synthesis are initiated. Again in this process; ovule development (Zhang and ONelll, 1993), swelling, development and coordination of male and female gametophytes (Zhang and ONelll, 1993) are provided in the ovary, while a change in curvature is observed in pedicel. In addition, stigmatic closure with auxin in the stigma, auxin promotion in the column and Ethylene production after pollination, RNA synthesis, synthesis of anythocyanin, swelling, straightening and greening are seen. With these, movement of substrates from the perianth to the ovary and column is observed (Arditti, 1969; Arditti et al., 1971; Arditti, 1979b). This post-pollination process is monitored in all orchid taxa, autogamic or allogamic.

Various research findings indicate that auxin and ethylene alone are not responsible for the post-pollination phenomenon of orchid flowersand enzymes, besides RNA and DNA are also effective in this process by spreading from the germinating pollen (Arditti 1979b). Thus, orchid pollinia and/or stigmas trigger a series of post-pollination events due to the synthesis or release of these substances in addition to auxin (Strauss and Arditti, 1982).

Pollen has auxin (Arditti 1979a). Although auxin is secreted from pollen, some researchers have emphasized that other factors affecting post pollination should be examined (Strauss and Arditti, 1982).

Therefore, in our first trials, the presence of Salicylic acid, Abscisic acid and Jasmonic acid hormones have been detected in polinia, apart from auxin. Because different studies have also referred to the presence of JA in pollen (Yamane et al., 1982). For this reason, it has been noted that there is a lack of knowledge in the form of whether there are other hormones in flowers after post-pollination and to what extent the activities of these hormones change in pollination between different taxa. For this reason, the first aim of the study is to investigate the hormones that could not be detected before during post pollination, its secondary objective is to determine the activities of newly discovered and lesser-known hormones such as SA and ABA in pollination experiments performed within the same taxon or with different taxa.

2. Material and Method

In the study, two different pollination experiments have been conducted: 1. Compatible pollination made with members of the same taxon (Intraspesific= ISP), 2. incompatible cross pollination experiments have been carried out with specimens from different genera (Intergeneric= IGP) to prevent fertilization as much as possible. As material, samples belonging to *Himantoglossum robertianum* (Loisel.) P. Delforge and *Orchis italica* Poir. have been used in the experiments (Figure 1a-d). The samples were collected from Muğla and Çanakkale in the previous year, some of them were dried and recorded in the herbarium (EDTU 22869, 10417), and some of them were kept in pots. Manual pollination experiments were carried out on specimens blooming in March the following year (Figure 1a-d). In order to confirm the accuracy of the samples, they were also identified (Deniz, 2022; Güler, 2022).

For this reason, polynariums taken from *H. robertianum* flowers (1 or 2 day-old flowers that have just opened and are suitable for pollination) by hand were placed on the stigmas of the flowers of the same taxon with the help of needle and forceps. Additionally, some polynariums from *O. italica* were also applied to another *H. robertianum* flower stigma. Therefore, both in-species pollination and hybridization between different genera were tested (Figure 1e-f). The ovaries with the stigma of the flowers taken from both types of pollination for 10 days were taken into liquid nitrogen and then kept in depfreeze at -80 °C until hormonal tests.

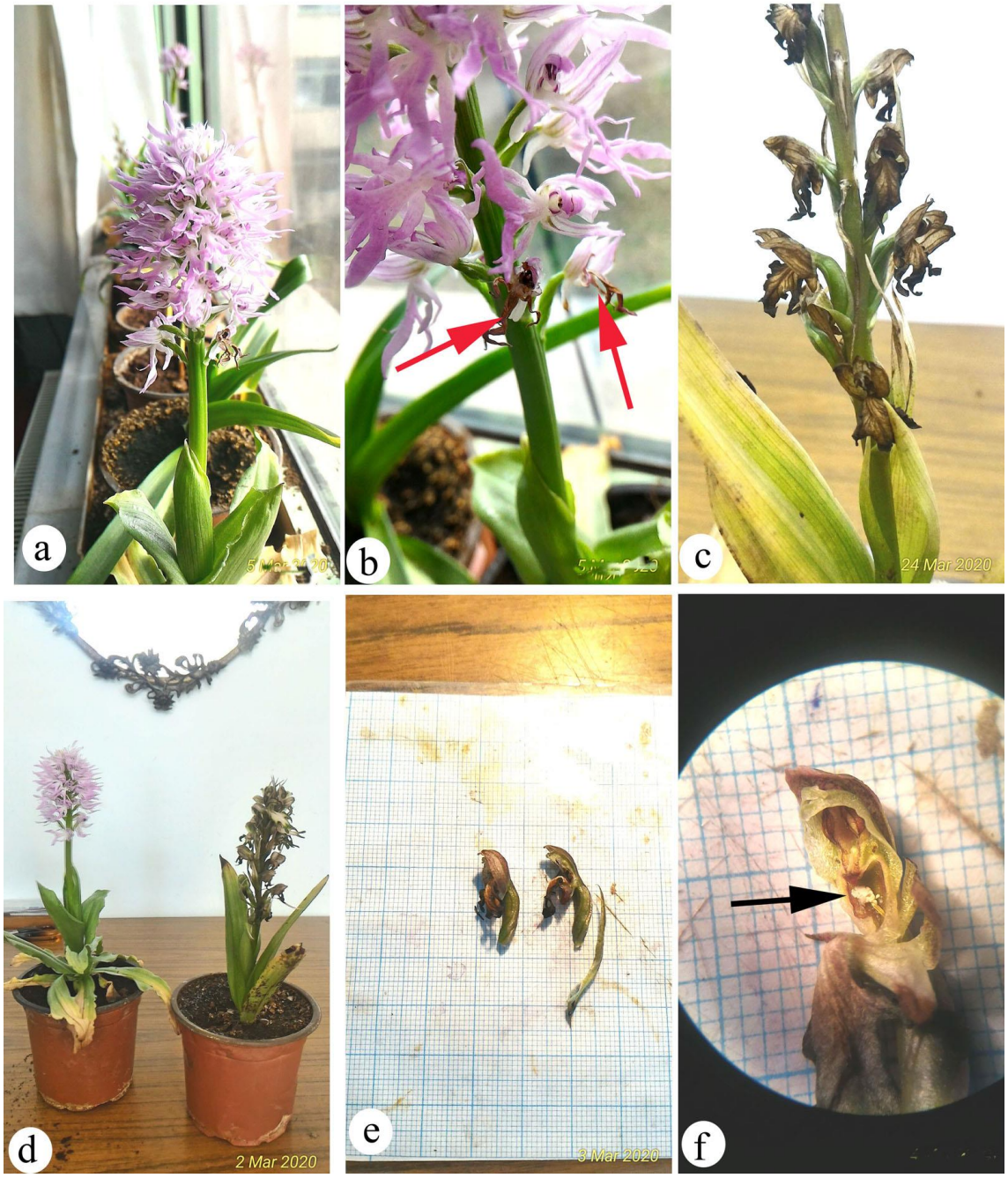

Figure 1: a. *O. italica* general view and, **b**. blackened labellums after pollination (red arrows), **c**. *H. robertianum* flowers and, **d**. *O. italica* and *H. robertianum* trial samples, **e.** dissected flowers of *H. robertianum* for hormone analysis, **f**. a polynarium (arrow) from *O.italica* on *H. robertainum* stigma.

Extracts were prepared according to the procedure of Müller and Munné-Bosch (2011) with some modification. The samples were pulverized using a grinder. After that, powdered samples were dissolved in 10 mL ethanol (95%) followed by ultrasonic assisted extraction in an ultrasonic cleaning bath for 60 min at 40 °C. This mixture was centrifuged at 5000 rpm for 30 min at +4 °C and the supernatant was collected into a volumetric flask. Extraction procedure was repeated twice. 5 mL ethanol was added to

the sample again and ultrasonic bath was performed at 40 °C for 30 min and centrifuged. Finally, the supernatants were combined into a 25 mL volumetric flask and the volume was made up to the mark with ethanol (95%). 100 µL of sample was mixed with 900 µL extraction solution (water, methanol, formic acid: v: v: v, 79: 20: 1), and samples were vortexed for 30 s. After that, the mixture was homogenized using sonicator at 45 °C 10 min. Samples were centrifuged at 13500 rpm for 5 min and supernatant injected into the LC–MS/MS system for quantitative analysis.

LC was performed using an Agilent 6460 (Agilent Technologies, Waldbronn, Germany) LC system. Data acquisition and processing were accomplished using MassHunter, the Agilent LC-MS software (Fischer et al., 2011). The concentration of the hormons in each sample was calculated using the calibration curve. Samples were prepared on the same day and analyzed in the same analytical run. All calibration curves were prepared with the following concentrations: blank (water, methanol, formic acid: v: v: v, 79: 20: 1), 5, 10, 25, 50, 100 ng / mL and injected all points three times. The linearity of all the hormone was $R2 \geq 0{,}995$. These samples were analyzed according to the procedure described for sample preparation.

Hormone analysis was performed by using an LC system (Agilent Technologies, Waldbronn, Germany). MS/MS analyses were conducted on an Agilent 6460 triple quadruple LCMS equipped with an electrospray ionization interface. 1 g sample was taken into falcon and on it 10 mL extra-pure water was added. The solution was vortexed for 1 min and sonicated for 15 min at 45 °C. BB and BP samples were centrifuged for 5 min at 13500 rpm. Then, 50 mL clear supernatant was mixed with 50 mL internal standard and 900 mL extraction solution (mobile phase A, methanol, acetonitrile: v :v :v, 5: 15: 15, Mobile phase B Acetic acid (1%) in 1:1 acetonitrile-methanol), and the sample was injected to LC-MSMS system. High-performance liquid chromatography parameters: Column Agilent Zorbax SB-C8 column 150 mm × 3,0 mm, 3,5 µm; particle size (Agilent Technologies). Autosampler temperature 4 °C, Flow rate 0,7 ml·min-1, Column temperature 35 °C, Injection volume 10 µl, Total run time 12 min. Mass spectrometry parameters: Ionization mode Electrospray ionization negative, Gas temperature 300 °C, Gas flow 10 l·min-1, Nebulizer 275 790,29 Pa, Sheath gas temperature 400 °C, Sheath gas flow 10 l·min-1, Capillary voltage 3 500 V, Nozzle voltage 0 V, Scan type Dynamic multiple reaction monitoring.

In each daily experiment, samples were taken from the flowers of a total of 6 different plants, 3 for ISP pollination and 3 for IGP pollination, and both types of 10-day trials were repeated three times and the differences in the hormonal values were compared using ANOVA with means separation by the Duncan's test using the SPSS 26 software at a significance level of $P \leq 0.05$.

3. Results and Discussion

In SA analyses, the highest ISP values were ISP1 and ISP3 (Table 1), and these values were significantly higher than ISP5,7,8 and 10 (Table 2). Although ISP4 and ISP9 values were also partly high, this increase was not significant (Tables 1-2). In SA's IGP experiments, the highest value was in IGP9,

followed by IGP4, IGP1, and IGP10, respectively, but there is no significant difference between the values (Tables 1,3).

When ABA values were considered, the highest ISP value was in ISP9, followed by ISP4, 1, 10, 6, and 5, respectively, and all comparisons with ISP9 were significant (Tables 4,5). In ABA, the highest IGP value was in IGP5, followed by IGP3, 6, and 4, respectively, and these high values in IGP3 and IGP5 were significant in comparisons (Tables 4,6).

In SA, ISP and IGP values were generally high, with almost similar values on days 1, 3, and 4. Additionally, the values on the 9th and 10th days of IGP also increased partially (Table 1). In ABA, IGP values were higher than ISP values, and only the ISP9 value was higher than all (Table 4).

Regarding the SA and ABA values in ISP, it is noteworthy that ABA values were generally higher than SA, except for ISP2–3 (Table 1.4). In IGP, ABA activities were higher at almost all values than in SA (Tables 1,4).

Table 1. Basic descriptives and Anova test results belonging to SA hormone activities in the all ISP and IGP groups

		95% Confidence Interval for Mean			
Groups	**N**	**Mean[a]**	**SD[b]**	**SE[c]**	**Sig.**
ISP1	3	2,4834	0,03755	0,02168	0
ISP2	3	1,8595	0,04407	0,02544	0
ISP3	3	2,4924	0,08805	0,05083	0
ISP4	3	2,056	0,182	0,10508	0
ISP5	3	1,5001	0,41715	0,24084	0
ISP6	3	1,8036	0,20813	0,12017	0
ISP7	3	1,4887	0,07332	0,04233	0
ISP8	3	1,2611	0,16519	0,09537	0
ISP9	3	1,9972	0,51759	0,29883	0
ISP10	3	1,6422	0,45481	0,26259	0
IGP1	3	2,2167	0,0583	0,03366	0,131
IGP2	3	1,7056	0,12711	0,07339	0,131
IGP3	3	2,0482	0,18857	0,10887	0,131
IGP4	3	2,4742	0,52223	0,30151	0,131
IGP5	3	1,6239	0,06184	0,0357	0,131
IGP6	3	1,8196	0,09824	0,05672	0,131
IGP7	3	1,3545	0,22917	0,13231	0,131
IGP8	3	1,3852	0,20125	0,11619	0,131
IGP9	3	3,3163	2,09457	1,2093	0,131
IGP10	3	2,1167	0,93076	0,53737	0,131

[a]: ng/gr; **[b]**: Standard deviation, **[c]**: Standard Error, ***F***: 6,599; ***df*: 9.**

Considering all ISP and IGP values, ABA IGP values on days 2, 3, 4, 5, and 6 were higher than all other ISP and SA IGP values on the same days, except the ABA ISP9 value, which is at the top of all other hormonal values (Tables 1, 4). JA was not found in any of the analyses.

Table 2. Mean comparison of SA activities in ISP experiments based on Duncan's test.

			Subset for alpha = 0.05			
	Grup	N	1	2	3	4
Duncan[a]	ISP8	3	1.2611			
	ISP7	3	1.4887	1.4887		
	ISP5	3	1.5001	1.5001		
	ISP10	3	1.6422	1.6422	1.6422	
	ISP6	3		1.8036	1.8036	
	ISP2	3		1.8595	1.8595	
	ISP9	3		1.9972	1.9972	1.9972
	ISP4	3			2.0560	2.0560
	ISP1	3				2.4834
	ISP3	3				2.4924
	Sig.		.137	.059	.115	.057

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table 3. Mean comparison of SA activities in IGP experiments based on Duncan's test.

			Subset for alpha = 0.05	
	Grup	N	1	2
Duncan[a]	IGP7	3	1.3545	
	IGP8	3	1.3852	
	IGP5	3	1.6239	
	IGP2	3	1.7056	
	IGP6	3	1.8196	
	IGP3	3	2.0482	2.0482
	IGP10	3	2.1167	2.1167
	IGP1	3	2.2167	2.2167
	IGP4	3	2.4742	2.4742
	IGP9	3		3.3163
	Sig.		.130	.077

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

Table 4. Basic descriptives and Anova test results belonging to ABA hormone activities in all ISP and IGP groups

95% Confidence Interval for Mean					
Groups	**N**	**Mean[a]**	**SD[b]**	**SE[c]**	**Sig.**
ISP1	3	4.2916	0.9364	0.54067	0.024
ISP2	3	1.7215	0.7252	0.41869	0.024
ISP3	3	1.04	0.3629	0.20956	0.024
ISP4	3	4.4433	2.8011	1.61725	0.024
ISP5	3	3.5281	0.2137	0.12342	0.024
ISP6	3	3.9603	1.4670	0.84701	0.024
ISP7	3	2.9267	0.91526	0.52843	0.024
ISP8	3	2.5784	1.99725	1.15311	0.024
ISP9	3	23.876	21.1418	12.20623	0.024
ISP10	3	3.8856	0.29605	0.17093	0.024
IGP1	3	2.0567	0.07105	0.04102	0.001
IGP2	3	7.0968	2.14384	1.23775	0.001
IGP3	3	11.115	7.59987	4.38779	0.001
IGP4	3	8.6526	0.42758	0.24686	0.001
IGP5	3	11.861	0.60766	0.35083	0.001
IGP6	3	9.3455	4.48871	2.59156	0.001
IGP7	3	1.318	0.87161	0.50322	0.001
IGP8	3	2.9797	0.05337	0.03082	0.001
IGP9	3	4.8108	0.6834	0.39456	0.001
IGP10	3	2.9254	0.66757	0.38542	0.001

[a]: ng/gr; **[b]**: Standard deviation, **[c]**: Standard Error, ***F***: 5,387; ***df*: 9.**

Table 5.Mean comparison of ABA activities in ISP experiments based on Duncan's test.

			Subset for alpha = 0,05	
	Grup	N	1	2
Duncan[a]	ISP3	3	1.0400	
	ISP 2	3	1.7215	
	ISP 8	3	2.5784	
	ISP 7	3	2.9267	
	ISP 5	3	3.5281	
	ISP 10	3	3.8856	
	ISP 6	3	3.9603	
	ISP 1	3	4.2916	
	ISP 4	3	4.4433	
	ISP9	3		23.8765
	Sig.		.597	1.000

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3.000.

Table 6: Mean comparison of ABA activities in IGP experiments based on Duncan's test.

			Subset for alpha = 0,05			
	Grup	N	1	2	3	4
Duncan[a]	IGP7	3	1.3180			
	IGP 1	3	2.0567	2.0567		
	IGP10	3	2.9254	2.9254		
	IGP 8	3	2.9797	2.9797		
	IGP 9	3	4.8108	4.8108	4.8108	
	IGP 2	3		7.0968	7.0968	7.0968
	IGP 4	3			8.6526	8.6526
	IGP 6	3			9.3455	9.3455
	IGP 3	3				11.1154
	IGP 5	3				11.8617
	Sig.		.201	.069	.094	.085

Means for groups in homogeneous subsets are displayed.

a. Uses Harmonic Mean Sample Size = 3,000.

SA is a signaling factor generally related to hormone regulation and systemic resistance of plants, and SA has a key role in seed germination, seedling formation, cell growth, respiration, enhancement of enzyme activity, regulation of plant growth and development, interaction with other organisms, and photosynthesis under adverse environmental conditions. (Chen et al., 2012). In fact, it has been stated that SA has a regulatory role that potentially affects plant growth and productivity when applied (Martin-Mex et al., 2005). For example; Carrot has also been used in tissue culture media to promote somatic embryogenesis of *Coffea arabica*, *Astragalus adurgens* and *Avena nuda* (Hong et al., 2008). Because SA increases the expression of somatic embryogenesis proteins by inhibiting ethylene biosynthesis (Luo et al., 2001). Inasmuch as; according to Behrouzyar and Yarnia (2015), SA is a potent regulator that can effectively manage a variety of plant growth metabolism. In recent studies, it has been shown that SA is very effective in plant growth, development and even reproductive organs (Medina et al., 2017; Belt et al., 2018). Therefore, this wide range of effects of this hormone is due to the fact that SA is present in all organs of the plant and can be transported to different organs via phloem (Baktır, 2010). SA also has to do with flower functions: For instance; SA manages the metabolism related to thermogenicity in flowers in *Cycas*, Annonaceae, Araceae, Aristolochiaceae, Cyclanthaceae, Nymphaceae and *Palmae* taxa (Oçkun, 2013).

SA plays important roles in regulating pollen viability, flower fertility and the development of normal female flowers (Liu et al., 2019). It has even been demonstrated that reactive oxygen species (ROS) homeostasis and SA hormone positively affect spikelet development and pollen fertility (Zhou et al., 2021). Again, SA and its derivative, MeSA, played a role in pollen tube growth of *Camellia oleifera* (Lu et al., 2021). Some studies have shown that SA is closely related to pollination and fertilization. For instance, in the pollinated pistil of the pear, the amount of SA increased significantly and promoted in vitro pollen germination and pollen tube elongation, and significantly increased the fruit formation rate (Zhao et al., 2012). Therefore, it is not surprising that SA is also involved in the orchid pollination process, as shown in our findings, and is supported by the literature analyzes shown above. Besides, the first discovery of endogenous SA in post-pollination events in orchids was provided by these findings, and the levels of this hormone should be determined in future studies regarding the development of the ovule during the transformation process from the ovary to the fruit. Thus, all SA activity from pollination to fruit and seed development will be clearer.

ABA is an important plant hormone that regulates plant growth, dormancy and responds to challenges. Apart from these ABA also contributes widely to the differentiation of male and female flowers (Xiao et al., 2003), embryo development (Cheng et al., 2014), fruit development and maturation. (Zhang et al., 2018). For instance, Gao et al. (2015) reported that ABA is involved in the development of different flower sexes in melon. In fact, ABA also participates in the development of plant reproductive structures. For example; ABA has been implicated in promoting microspore-derived embryos in *Brassica napus* (Zou et al., 1995). In another study (Carrier et al., 1999), the highest ABA content was observed in the female gametophyte at the 11th day after pollination when cellular activities changed. In another study

(Vernieri et al., 1989), it was emphasized that ABA in the ovule, nucellus and integuments increased continuously at close levels and until anthesis and remained constant before pollination. ABA also has an effect on seed development and the development of sporophyte and gametophyte tissues (Chen et al., 2020).

ABA is also related to pollen sterility. For instance, ABA levels in sterile orange blossoms were higher than normal pollen (Harris and Dugoer, 1986). Additionally, ABA is also effective on pollen activity. Thus, ABA level of the stigma during pollination is very important in the process from the adhesion of pollen grains to their germination (Kovaleva and Zakharova, 2003). For instance, pollen tube plasma membrane H^+ concentration and related ATPase activity are related to Ca^+ and K^+ channels and by increasing the activity of free Ca^+ and reactive oxygen species (ROS) and H^+-dependent ATPase of ABA in the pollen tube, it contributes to the elongation of the pollen tube (Bright et al., 2009). Additionally, ABA also modulates the activity of other hormones, such as ethylene, to regulate pollen germination and tube elongation prior to fertilization (Firon et al., 2012). It was also stated that ABA is important in incompatible pollination (Barendse et al. 1970). As a result, in the light of the detailed literature review above, the fact that ABA hormone, which is effective at every stage from the flowering process, the functions of the reproductive organs, the pollen and seed, fruit development process to their activities and infertility, parthenocarpy, compatible and self-incompatible pollination states, showed different activities in ISP and IGP experiments in our studies is not surprising. As a matter of fact, in post-pollination studies (Arditti et al., 1971), the effect of ABA was investigated by external exogenous application, and endogenous hormone amount analysis was not performed. According to the study of Arditti et al. (1971), it was stated that exogenous ABA application only increased anthocyanin synthesis in the post-pollination process. However, according to our study, the values of ABA in ISP pollination were generally lower than in IGP pollination (Table 4). At this point, as stated by Goldschmit (1980) and (Kojima et al., (1993), ABA is important in the process of pollen germination in the stigma and especially in the progression of the pollen tube in the stylus in compatible pollination and it has been stated that it promotes this situation. Likewise, ABA amounts have always remained at high levels in incompatible pollination (Barendse et al. 1970). In addition, as indicated in Table 4, the fact that the amount of endogenous ABA is higher in IGP pollination, especially between 2-6 days and partially on the day 9, compared to ISP, indicates that this hormone ceases the pollen tube progression and controls the stylus functions in this direction, and thus the ovary remains stable before fertilization. This indicates the importance of ABA in keeping it in a state (Hu et al., 2019). In berief, ABA prevented fertilization with its high values in IGP pollination, while it was an incentive at low values in ISP. ABA realized this especially with her functions in the stigma and stylus. Thus, Cheng et al. (2002) suggested that ABA has dual functions as inhibitor and activator and emphasized that low endogenous ABA levels are activating this hormone. It is seen that our findings are compatible with the detailed literature data given above and ABA is notably active in the post-pollination process in orchids. Naturally, supporting the development of the stylus, stigma and ovule simultaneously with embryological studies will increase

the accuracy of these ideas. As a matter of fact, the ABA ISP9 value is the highest hormonal value, and additional embryological histological studies will reveal why ABA increases significantly on the 9th day of ISP.

During our literature analysis, no cross pollination experiments such as IGP were encountered in post-pollination studies in orchids. Therefore, our findings represent a novelty as they are aimed at detecting both endogenous hormone levels and SA activity and ABA activity for the first time in both pollination experiments. Therefore, it our original findings here have revealed that it will be possible to understand the natural responses of the plant to pollination by monitoring endogenous hormone levels. In our future studies, the activity of these hormones will be better understood and contribute to the reproductive biology of orchids by examining the expression levels of some enzyme genes in the post-pollination process together with embryological studies. SA has many functions in flower development, including regulation of stamen development, flowering time, and ovary development (Luo et al., 2022). SA regulates pollen tube elongation during pollination; namely; regulates local pollen tube growth depending on SA content in plant pistils (Lu et al., 2021). Also, pear pollen tubes showed that stamens produced SA in large amounts after pollination to promote pollen tube germination and pollen tube elongation (Luo et al., 2022). SA regulates ovarian development, episperm and endosperm development. after fertilization. Also Sa effected stress-induced flowering and flower development. The association between plant immunity and the flowering process has been studied in *Arabidopsis*, where pathogen attack suppresses the nuclear factors YB2 and YB3 to induce the endogenous hormones SA and Jasmonic acid (JA) which then promote flowering (Xie, 2021). It was found by direct pollination extraction that IAA is related to ABA, SA, and some GAs during the post-pollination process. In this study, hormones in the polynia were studied, but hormones in the ovary were not examined (Netlak et al., 2022). In our study, hormonal changes after pollination were detected for the first time in the scientific world. In another study, JA was found to activate the self-incompatibility mechanism and to do this especially in the ovary. (Liu et al., 2022). For pollination drop in Ginkgo biloba, DEGs (differentially expressed genes) related to jasmonic acid (JA) metabolism and signal transduction were identified (Wanqing et al., 2021). Seedless fruit is a feature appreciated by consumers, and it has been found that GA, JA, ethylene, and SA are active together in the ovule abortion process for seedless fruit in some plants (Wang et al., 2021). JA activity has been found to be important in stamen filament elongation in *Arabidopsis* (Zhao et al., 2021). The plants whose brief analysis is given above are plants other than orchids. To date, the findings mentioned in this article have not been observed in any orchid, including *Himantoglossum* spp.

Conclusions

In SA analyses, the highest ISP values were ISP1 and ISP3, and these values were significantly higher than ISP5,7,8 and 10. Although ISP4 and ISP9 values were also partly high, this increase was not significant. In SA's IGP experiments, the highest value was in IGP9, followed by IGP4, IGP1, and

IGP10, respectively, but there is no significant difference between the values. Also in SA, ISP and IGP values were generally high, with almost similar values on days 1, 3, and 4. Additionally, the values on the 9th and 10th days of IGP also increased partially. In ABA analyses, the highest ISP value was in ISP9, followed by ISP4, 1, 10, 6, and 5, respectively, and all comparisons with ISP9 were significant. The highest IGP value was in IGP5, followed by IGP3, 6, and 4, respectively, and these high values in IGP3 and IGP5 were significant in comparisons. Also In ABA, IGP values were higher than ISP values, and only the ISP9 value was higher than all.

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Compliance with ethical standards

The author declare that they have no conflict of interest. This article does not contain any studies involving animals or human participants as objects of research.

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