Effect of Phenolic Acids on *in vitro* Pollen Germination of Olive and Date Palm

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ABSTRACT

The effects of phenolic acids (benzoic, caffeic, ferulic, gallic, gentisic, homovanillic, p-hydroxybenzoic, protocatechuic, pyrogallic, shikimic, syringic, and vanillic acids) on *in vitro* germination were evaluated on pollen from olive and date palm. In addition to immersing olive pollen in olive oil, adding benzoic and vanillic acids (50, 100, 150 µM), ferulic acid (100 µM), caffeic acid (100, 150 µM), and gallic acid (200, 400 µM) to the basic germination medium (0.8% agar, 10% sucrose and 800 µM boric acid) has significantly improved *in vitro* pollen germination. In contrast, immersing date palm pollen in olive oil has inhibited *in vitro* pollen germination. Moreover, adding phenolic acids at any concentration to the basic germination medium (1% agar, 8% sucrose and 100 µM citric acid) did not improve pollen germination. Finally, high concentrations of phenolic acids have inhibited *in vitro* pollen germination of both species.

Keywords: Agar media, *In vitro* pollen germination, *Olea europaea*, Phenolic acids, *Phoenix dactylifera*.

INTRODUCTION

Olive (Olea europaea L.) is widespread in most Mediterranean countries, Argentina, Australia, Iran, United States of America, Chile, and Peru while date palm (Phoenix dactylifera L) is widespread in Middle East and North African countries, Pakistan, China, Albania, and United States of America. In Jordan, olive is the first fruit crop and represents about 70% of the cultivated area to fruit crops, while date palm is one of the promising fruit crops and the cultivated area has been increased five folds in the last decade (FAO, 2012).

Olive is andromonoecious, i.e. individual tree bears both hermaphroditic and staminate flower, while date palm is dioecious, i.e. has separate female and male trees. In both crop species, economic yield is determined mainly, by a successful pollination and fertilization. Cross or artificial pollination are usually essential to overcome pollination problems. In date palm orchard, artificial pollination is essential to produce commercial crop, manual pollination is tradition, while mechanical pollination is the most promising for commercial production (Al-Wusaibai *et al*., 2012; Awad, 2010; Khalil and Shawaan, 1982). While mechanical pollination is practiced, pollen either is mixed with wheat flour or diluted in sucrose solution, both should have no harmful substances on pollen viability (Zaid and de Wet, 2002). In olive orchard, cross pollination is usually essential to overcome pollination problems (Ateyyeh *et al*., 2000; Cuevas *et al*., 2009; Guerin and Sedgley, 2007).

There are a number of factors that influence pollen germination and pollen tube growth which can be summarized into three categories: incongruity “no reaction”, incompatible interaction, and compatible

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interaction (Lersten, 2004). With regard to compatible and incompatible situations, the interaction depends on the stigma type (wet or dry) - both species under study are of the wet type (Raghavan, 1997; Rejon *et al*., 2012; Serrano *et al*., 2008), which have surface cells that often lyse to release a viscous surface secretion containing proteins, lipids, polysaccharides, peptides, phenolic compounds, water and pigments (Edlund *et al*., 2004; Lersten, 2004). These components play a vital role in post-pollination processes including pollen adhesion, hydration during germination and pollen tube entry into the stigma (Shivanna, 2003). In addition, phenolic compounds on and within the stigma may play an important role in passive inhibition; as they selectively inhibit or promote germination. In oil palm, the stigmatic groove and stylar canal contain an extracellular matrix secreted by the canal cells which is rich in proteins, acidic polysaccharides and pectins (Tandon *et al*., 2001). Serrano *et al*. (2008) found that the extracellular components of the olive stigmatic receptive surface are heterogeneous, including carbohydrates, lipids and proteins.

Pollen itself can release a wide range of metabolites into a culture medium. These include sugars, amino acids, proteins, phenolic compounds, RNA, and polyamines (Shivanna, 2003). In addition, phenolic compounds such as quercetin, kaempferol have been found in the pollen of *Phoenix sylvestris*, *Areca catedru* and *Cocos tirtcifera* (Bhattacharya *et al*., 1993).

The role of phenolic compounds in the plant's reproductive system has been studied. Pollen fertility as well as flavonoid synthesis was disrupted in petunia and maize mutants, with both mutants being self-sterile due to a failure to produce a functional pollen tube. Adding micromolar quantities of kaempferol to the germination medium or to the stigma at pollination was sufficient to restore normal pollen germination and tube growth *in vitro* and full seed set *in vivo* (Mo *et al.*, 1992). Compounds that promoted the growth of pollen tubes were isolated from the style of *Rhododendron mucronatum* and were identified as azalein, (+)-catechin, and (-)-epicatechin (Ozawa *et al*., 1993).

In a study to identify the best pollen germination medium for olive, Ateyyeh *et al.* (2000) found that immersing olive pollen in olive oil improved *in vitro* pollen germination, this result indicated that there was a major factor in olive oil that affected this process. Olive oil is very rich in phenolic acids such as benzoic acid, caffeic acid, ferulic acid, gallic acid, gentisic acid, homovanillic acid, *p*-hydroxybenzoic acid, protocatechuic acid, pyrogallic acid, shikimic acid, syringic acid, and vanillic acid (Boskou *et al*., 2006; García-Rodríguez *et al.*, 2015; Khadem and Marles, 2010; Tuck and Hayball, 2002). On the other hand, date palm pollen is mixed with wheat flour before dusted to female date palm inflorescences, wheat flour contained many phenolic acids such as ferulic, caffeic, syringic, and gentisic (Wang *et al*., 2013), but their effect on pollen germination and pollen tube growth did not studied before. Therefore, the current study was carried out to investigate the effect of phenolic acids on in vitro pollen germination of olive and date palm.

MATERIALS AND METHODS

Plant material

Olive pollen was collected from cultivar 'Nabali Baladi' trees on University of Jordan campus located at a latitude 32^{o} 0' N, a longitude 35^{o} 52' E, and an altitude of about 1000 m. While date palm pollen was collected from cultivar 'Barhi' trees located at University of Jordan Agricultural Research Station in the Jordan valley located at a latitude 32^{o} 05' N, a longitude 35^{o} 35' E, and an altitude of about -267 m. The experiment was conducted in the laboratories of Department of

Horticulture and Crop Science, Faculty of Agriculture, The University of Jordan.

Pollen collection and storage

Inflorescences of date palm and olive were collected during May 2010. The flower buds were at the development stage preceding anthesis. The inflorescences were spread over white paper on the bench in the laboratory under room temperature ($24^{o}C\pm2$) for 1 day, allowing anthers to dehisce. Pollen of each species was then separately collected into glass tubes. The collected pollen was dehydrated in the open glass tubes for 4 hours using desiccators partially filled with dry silica gel, and then dehydrated pollen was placed in twelve cryotubes for each species then it were subsequently sealed and plunged directly in liquid nitrogen for at least 15 minutes. Thereafter, cryotubes were stored in a deep freezer at –80 °C.

Pollen viability test

A 2, 3, 5–triphenlytetrazolium chloride (TTC) stain test was used before germination test to ensure that pollen had not lost its viability. Combinations of 1% TTC and 30% sucrose for date palm pollen (Ateyyeh, 2012), and 1% TTC and 60% sucrose for olive pollen (Ateyyeh, 2009) were used for that test. One drop of TTC-sucrose solution was placed on a microslide, then a small amount of pollen was suspended in that drop and a cover glass was placed onto the microslide. The covered microslide was wrapped with aluminum foil and then incubated in the chamber room at 30 ± 2 °C for 60 min. About one hundred pollen grains were counted under a light microscope to determine viability percentage.

***In vitro* pollen germination test**

A preliminary experiment was conducted to find the best basic germination medium for both species. Eight concentrations (treatments) of boric acid (0, 400. 500, 600, 700, 800, 900 and 1000 µM) and nine concentrations (treatments) of citric acid (0, 50, 100, 150, 200, 250, 300, 350 and 400 µM) were used with 0.8% agar and 10% sucrose for olive pollen and with 1% agar and 8% sucrose for date palm pollen. Each treatment was replicated five times, the replicate consists of one petri-dish and two hundred pollen were counted per petri-dish. Accordingly, the basic germination medium contained 0.8% agar, 10% sucrose, and 800 µM boric acid for olive. While for date palm, the basic germination medium contained 1% agar, 8% sucrose, and 100 µM citric acid (Table 1).

Pollen germination of olive was tested *in vitro* on nine germination media (treatments) for each of the following phenolic acids (benzoic, caffeic, ferulic, gallic, gentisic, homovanillic, *p*-hydroxybenzoic, protocatechuic, pyrogallic, shikimic, syringic, and vanillic acid). The control treatment was the basic germination medium comprising 0.8% agar, 10% sucrose, and 800 µM boric acid (treatment 1). For treatment with olive oil, pollen was immersed in olive oil before spreading it over the basic medium (treatment 2). The phenolic acid treatments consisted of the basic germination medium and one of the following concentrations of one of the phenolic acids 50, 100, 150, 200, 400, 600, and 800 µM (treatments 3-9). Date palm pollen germination was tested *in vitro* on the same nine germination media (treatments) as olive. However, the basic germination medium consisted 1% agar, 8% sucrose, and 100 µM citric acid.

Each species was done separately. Each treatment was replicated five times in a completely randomized design for each phenolic acid, the replicate consists of one petri-dish and two hundred pollen were counted per petri-dish. In all cases, the petri-dishes were covered with aluminum foil, and then incubated in a chamber

room at 27 ºC for 12 hours. The number of germinated pollen was counted under a light microscope at (10X).

Data analysis

Statistical Analysis System Package (SAS Institute Inc., 2009) was used to analyze the results. Pollen germination percentages were analyzed by one way analysis of variance. Comparisons among the treatments were carried out by LSD test. Least squares means were sorted with the pdmix800 macro (Saxton, 1998).

RESULTS

Olive

Pollen viability exceeded 90% before each experiment. The basic germination medium contained 0.8% agar, 10% sucrose and 800 µM boric acid; it had germination percentage significantly (33.4%) higher than the other treatments (Table 1). Pollen germination was significantly improved by the following treatments: immersion of pollen in olive oil (76.8%); benzoic acid at 50, 100, and 150 µM (75.1, 74.8 and 77.0%, respectively); caffeic acid at 100, and 150 µM (76.5and 77.0%, respectively); ferulic acid at 100 µM (79.7%); gallic acid at 200, and 400 µM (79.2 and 79.7%, respectively); and vanillic acid at 50, 100, and 150 µM (77.2, 80.0 and 78.9%, respectively) (Table 2). Pollen tube length was not measured in this experiment but it was obvious that pollen tubes treated with these phenolic acid concentrations were longer than those in the control and olive oil treatments. Other phenolic acids (gentisic acid, *p*-hydroxybenzoic acid, protocatechuic acid, and shikimic acid) improved pollen germination but not to the same level. Germination percentage was completely inhibited at 50 µM and higher concentrations of syringic acid; at 200 µM and higher concentrations of ferulic acid and vanillic acid; at 400 µM and higher concentrations of benzoic acid, homovanillic acid and pyrogallic acid; at 600 µM and higher concentrations of gallic acid and *p*-Hydroxybenzoic acid; at 800 µM and higher concentrations of caffeic acid and gentisic acid (Table 2).

Date Palm

Pollen viability exceeded 90% prior each experiments. The basic germination medium contained 1% agar, 8% sucrose, and 100 µM citric acid; it had germination percentage (89.8%) significantly higher than the other treatments (Table 1). Pollen germination was very high (90.0-92.7%) on the basic germination medium (control). Immersing pollen in olive oil, before spreading it on the medium, completely inhibited pollen germination. Further, adding any phenolic compound to the medium did not improve pollen germination. Germination was completely inhibited at 200 µM and higher concentrations of caffeic acid, ferulic acid and vanillic acid; at 400 µM and higher concentrations of benzoic acid, *p*-Hydroxybenzoic acid, homovanillic acid and pyrogallic acid; at 600 µM and higher concentrations of gallic acid, gentisic acid and protocatechuic acid; and at 800 µM and higher concentrations of shikimic acid and syringic acid and (Table 3).

DISCUSSION

The results of this experiment emphasized that phenolic acids play an important role in pollen germination Phenolic compounds, including phenolic acids are allelochemicals released from pollen and interfered with pollen germination (Kyogoku, 2015; Loughnan *et al*., 2014; Murphy *et al*., 2009; Roshchina, 2001; Roshchina, 2009; Shraddha *et al*., 2007)). Also the results show that the effect of these phenolic acids varied depending on concentrations and species, since adding benzoic and vanillic acids (50, 100, 150 µM), ferulic acid (100 µM), caffeic acid (100, 150 µM), and gallic acid (200, 400 µM) to the basic germination medium has

significantly improved *in vitro* pollen germination of olive pollen but did not improve pollen germination of date palm. In other studies, very low concentrations of the phenolic compounds had strongly promoted *in vitro* pollen germination of tobacco (Ylstra *et al*., 1992), and *Camellia japonica*, *Rhododendron mucronatum*, *Styrax japonica* and *Pinus densiflora*, but had no significant effects on *Lilium auratum* and *Narcissus pseudo-narcissus* (Ozawa *et al*., 1993)

Unfortunately, the exact biological mechanism of phenolic compounds on pollen germination is unknown (Forbes *et al*., 2014). However, the mechanisms of action of phenolic compounds on other biological activities have been summarized by John and Sarada (2012) who mentioned that phenolic compounds exert allelopathic effects on various physiological processes in plants such as inhibition of cell division, elongation, and submicroscopic structures, changes in membrane permeability and inhibition of plant nutrients uptake, plant photosynthesis and respiration, various enzymes functions and activities, synthesis of plant endogenous hormones and protein synthesis.

The inhibitory effect of high concentration of phenolic acids on pollen germination shown in this study should be taken into consideration during practices involving artificial pollination. For example, pollen of date palm is typically mixed with white wheat flour for artificial pollination (Al-Wusaibai, 2012; Khalil and Al-Shawaan, 1982) and wheat flour contains many phenolic acids such as ferulic, caffeic, chlorogenic, syringic, gentisic, and *p*-coumaric acid (Wang *et al*., 2013). Usually, farmers get variable results and, therefore, pollinate trees 2 to 4 times to ensure sufficient fruit set and then thin out the excess fruits. To improve date palm fruit set, date palm pollen should be suspended in either sugar solution (Khalil and Al-Shawaan, 1982) or in water (Awad, 2010). Moreover, biotic stresses such as those from diseases and insects (Ruelas *et al*., 2006; Taware *et al*., 2010) and abiotic stresses (drought, minerals deficiency, shade) (Gallagher *et al*., 2010; Karageorgou *et al*., 2002) can induce high concentrations of phenolic acids in the plant, so avoiding such stresses may enhance pollen germination, pollen tube growth and fruit set. Many olive groves located in arid region with hot dry climate and infertile saline alkaline soil (Bainbridge, 2007), where pollen germination and pollen tube growth were restricted and self-incompatibility problem was increased (Ayerzar and Coates, 2004; Guerin and Sedgley, 2007; Koubouris *et al*., 2009). Under such conditions, hand- and mechanical-pollination with good viable pollen overcome the problem (Ayerzar and Coates, 2004). Further studies concerning the relationship between phenolic acids and the pollen - stigma interaction are needed to better understand the mechanisms involved.

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Table 1. Effect of boric acid and citric acid on *in vitro* olive pollen germination percentage.

Concentration µM	Pollen germination %	
	olive	date palm
0.0 (Control)	0.00 g[z]	15.3 j[z]
Boric acid		
400	0.00 g	68.1 d
500	7.30 f	62.6 e
600	18.7 de	73.7 c
700	25.1 c	70.8 cd
800	33.4 a	78.8 b
900	24.2 c	55.2 g
1000	22.6 cd	33.4 h
Citric acid		
50	0.00 g	61.0 ef
100	0.00 g	89.8 a
150	0.00 g	80.1 b
200	10.0 f	57.6 fg
250	29.7 b	33.9 h
300	24.8 c	19.7 i
350	20.3 d	0.00 k
400	15.3 e	0.00 k

[z] Means in the same column having the same letters are not significantly different at P=0.05.

Table 2. Effect of different concentrations of phenolic acids on *in vitro* olive pollen germination percentage

	Treatments							
Phenolic acids	**Control**	**Olive oil**	**50 µM**	**100 µM**	**150 µM**	**200 µM**	**400 µM**	**600 µM**	**800 µM**
1[y]	31.7 c[z]	76.8 a	75.1 a	74.8 a	77.0 a	55.3 b	0.0 d	0.0 d	0.0 d
2	30.8 e	75.1 a	53.0 c	76.5 a	77.0 a	57.2 b	47.9 d	27.0 e	0.0 f
3	32.4 b	77.0 a	33.3 b	79.7 a	21.0 c	0.0 d	0.0 d	0.0 d	0.0 d
4	31.5 b	78.6 a	26.0 c	20.6 d	32.0 b	79.2 a	79.7 a	0.0 e	0.0 e
5	29.0 de	74.8 a	18.1 f	27.8 e	32.9 cd	42.3 b	40.2 b	35.4 c	0.0 g
6	28.7 b	73.6 a	28.8 b	15.3 c	14.8 c	29.0 b	0.0 d	0.0 d	0.0 d
7	29.5 d	75.0 a	29.2 d	28.0 d	27.8 d	36.0 c	43.3 b	0.0 e	0.0 e
8	31.1 de	74.4 a	0.0 f	28.6 e	33.6 d	31.8 de	56.3 b	40.4 c	30.7 de
9	30.8 b	75.6 a	32.6 b	30.9 b	32.2 b	33.7 b	0.0 c	0.0 c	0.0 c
10	29.1 d	73.0 a	17.0 ef	15.0 f	46.5 b	30.0 d	38.4 c	35.8 c	20.3 e
11	30.0 b	73.2 a	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c	0.0 c
12	31.0 b	78.5 a	77.2 a	80.0 a	78.9 a	0.0 c	0.0 c	0.0 c	0.0 c

[z] Means in the same row having the same letters are not significantly different at P=0.05.

[y] (1=Benzoic, 2= Caffeic, 3=Ferulic, 4=Gallic, 5=Gentisic, 6=Homovanillic, 7= *p*-Hydroxybenzoic, 8=Protocatechuic, 9=Pyrogallic, 10=Shikimic, 11=Syringic, 12=Vanillic).

Table 3. Effect of different concentrations of phenolic acids on *in vitro* date palm pollen germination percentage.

	Treatments								
Phenolic acids	**Control**	**Olive oil**	**50 µM**	**100 µM**	**150 µM**	**200 µM**	**400 µM**	**600 µM**	**800 µM**
1	90.7 a [z]	0.0 d	83.2 c	83.7 c	85.6 bc	88.3 ab	0.0 d	0.0 d	0.0 d
2	91.7 a	0.0 d	86.0 b	80.0 c	80.3 c	0.0 d	0.0 d	0.0 d	0.0 d
3	91.0 a	0.0 d	82.6 c	87.7 b	80.7 c	0.0 d	0.0 d	0.0 d	0.0 d
4	91.3 a	0.0 d	91.3 a	93.0 a	89.7 ab	86.8 b	79.7 c	0.0 d	0.0 d
5	91.7 a	0.0 c	92.3 a	90.0 a	91.2 a	91.0 a	55.7 b	0.0 c	0.0 c
6	91.7 a	0.0 d	76.0 b	68.3 c	67.8 c	67.7 c	0.0 d	0.0 d	0.0 d
7	90.7 a	0.0 b	92.0 a	91.0 a	90.2 a	90.7 a	0.0 b	0.0 b	0.0 b
8	91.7 a	0.0 c	93.0 a	92.6 a	90.3 a	92.7 a	72.0 b	0.0 c	0.0 c
9	91.0 a	0.0 c	93.1 a	92.0 a	80.3 b	80.0 b	0.0 c	0.0 c	0.0 c
10	90.7 a	0.0 f	75.3 b	63.6 c	49.3 d	48.2 d	49.0 d	19.3 e	0.0 f
11	90.0 a	0.0 e	41.0 b	35.3 c	34.6 c	34.7 c	29.3 d	29.0 d	0.0 e
12	92.7 a	0.0 b	93.0 a	93.5 a	93.8 a	0.0 b	0.0 b	0.0 b	0.0 b

[z] Means in the same row having the same letters are not significantly different at P=0.05.

[y] (1=Benzoic, 2= Caffeic, 3=Ferulic, 4=Gallic, 5=Gentisic, 6=Homovanillic, 7= *p*-Hydroxybenzoic, 8=Protocatechuic, 9=Pyrogallic, 10=Shikimic, 11=Syringic, 12=Vanillic).

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تاثير الأحماض الفينولية على انبات حبوب لقاح الزيتون والنخيل في المختبر

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ملخص

جرى تقييم أثر الأحماض الفينولية التالية (بنزويك، كافيك، فيرولك، غالك، جنتسك، هوموفانيلك، مونوهايدروكسي بنزويك، بروتوكاتكويك، بايروغالك، شكمك، سيرينجك، فانيلك) على إنبات حبوب لقاح الزيتون و النخيل في المختبر. لقد تحسنت نسبة انبات حبوب لقاح الزيتون بشكل معنوي بعد غمرها في زيت الزيتون، بالإضافة إلى ذلك فإن إضافة الحمضين بنزويك وفانيلك بتركيز 50 و 100 و 150 ميكرومولر وحمض فيرولك بتركيز 100 ميكرومولر وحمض كافيك بتركيز 100 و 150 ميكرومولر وحمض غالك بتركيز 200 و 400 ميكرومولر إلى المكونات الأساسية لوسط الإنبات (0.8 ٪ أجار و 10٪ سكروز و 800 ميكرومولر من حمض بوريك) حسنت الإنبات المخبري لحبوب لقاح الزيتون معنويا. في المقابل، فإن غمر حبوب لقاح النخيل في زيت الزيتون ثبط الإنبات المختبري لحبوب اللقاح. كما أن إضافة أي تركيز من الأحماض الفينولية إلى المكونات الأساسية لوسط الإنبات (1٪ أجار، 8٪ سكروز و 100 ميكرومولر من حمض ستريك) لم تحسن الإنبات المختبري لحبوب لقاح النخيل. وأخيرا، فقد ثبطت اضافة التركيزات العالية من الأحماض الفينولية إنبات حبوب لقاح كلا النوعين.

الكلمات الدالة: الأحماض الفينولية، إنبات حبوب اللقاح في المختبر، أوساط انبات من الاجار، الزيتون، النخيل.

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