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Effects of Brassinosteroid, Naphthalene Acetic Acid and Gibberellic Acid on *In vitro* Pollen Germination of Bisexual and Functional Male Flowers of Pomegranate Cultivars

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Abstract

The two types of flowers (bisexual and functional male) on a pomegranate tree produce their own pollen. Having these different sources for pollen can enable growers or breeders to have an option for crossing purposes. With this point of view, pollen collected from both type of flowers of three cultivars, 'Antalya–14', 'Aşınar' and 'Çekirdeksiz', were tested for their ability to germinate *in vitro* on a medium (1% agar and 20% sucrose) containing three different group of hormones, gibberelic acid (GA_3), naphthalene acetic acid (NAA) and 24–epibrassinolide (EBR). After 24 hours of incubation, pollens were evaluated and germination ratios were determined. Germination ability of pollen from different flowers greatly differed depending on the cultivars. Bisexual flowers from 'Antalya–14' and 'Çekirdeksiz' had significantly lower ratio, but not in cv. 'Aşınar'. Effects of the hormones were mostly dependent on pollen source, type and concentration and also cultivar. GA_3 reduced the ratio in bisexual flowers but the effect on functional males was not in the same manner. NAA caused a decrease in all pollen germination. EBR on the other hand, generally increased it while this influence was mainly related with the flower type and cultivar. A deduction can be made as cultivars with high germination ability in their male flowers could ensure adequate pollination and/or can be used as pollinators for other cultivars.
Keywords: *Punica granatum* L., Epibrassinolide, Gibberellin, Auxin, Pollen germination

Öz

Nar Çeşitlerinin Erselik ve Fonksiyonel Erkek Çiçeklerinde *in Vitro* Polen Çimlenmesi Üzerine Brassinosteroid, Naftalen Asetik Asit ve Giberllik Asitin Etkileri

Bir nar ağacı üzerinde bulunan iki farklı tipteki çiçek (erselik ve fonksiyonel erkek) kendilerine ait polen üretmektedir. Bu şekilde farklı polen kaynaklarına sahip olmak, yetiştiricilere veya ıslahçılara melezleme açısından bir seçenek sunmaktadır. Bu bakış açısıyla, üç nar çeşidinin ('Antalya–14', 'Aşınar' ve 'Çekirdeksiz') her iki tip çiçeğinden toplanan polenler, içinde gibberellik asit (GA_3), naftalen asetik asit (NAA) ve 24–epibrassinolid (EBR) bulunan besin ortamı (%1 agar ve %20 sakkaroz) içerisinde *in vitro* çimlenme yetenekleri açısından test edilmiştir. 24 saatlik inkübasyondan sonra polenler değerlendirilmiş ve çimlenme oranları saptanmıştır. Farklı çiçeklerden elde edilen polenlerin çimlenme yetenekleri çeşide göre büyük farklılık göstermiştir. 'Antalya–14' ve 'Çekirdeksiz' çeşitlerinin erselik çiçekleri önemli düzeyde düşük orana sahip olurken 'Aşınar' çeşidinde bu durum gözlenmemiştir. Hormonların etkileri büyük çoğunlukla polen kaynağı, hormon tipi ve konsantrasyonu ile çeşide bağlı olmuştur. GA_3 erselik çiçeklerde oranı düşürmekle beraber fonksiyonel erkek çiçeklerde bu etki aynı yönde olmamıştır. NAA bütün polenlerin çimlenmesinde azalmaya neden olmuştur. Öte yandan EBR genel olarak arttırdıysa da bu etki esas olarak çiçek tipi ve çeşit ile ilişkili olmuştur. Fonskiyonel erkek çiçeklerinde yüksek çimlenme yeteneği olan çeşitlerin yeterli tozlanmayı güvence altına alacağı ve/veya diğer çeşitleri için tozlayıcı olarak kullanılabilecekleri düşünülebilir.
Anahtar kelimeler: *Punica granatum* L., Epibrassinolid, Giberellin, Oksin, Polen çimlenmesi

Introduction

In vitro pollen germination tests are one of the valuable tools used in identification of genotypic differences between cultivars in a species (Gadze et al., 2011). Literature on *in vitro* pollen germination of horticultural crops is abundant with studies determining basic medium constituents including boric acid, mineral salts or plant growth regulators. Since their discovery, plant growth regulators, or hormones are linked to or found responsible in plant physiology and growth responses and there are contradicting results on their effects when added to the growth medium. Some researchers showed that auxins and gibberellins generally promote or inhibit pollen germination and growth (Vitagliano and Viti, 1989; Viti et al., 1990), whereas inhibition occurred when cytokinins and abscisic acid were utilized (Hewitt et al., 1985; Tosun and Koyuncu, 2007). Acar et al. (2013) reported reduced *in vitro* pollen germination in male flowers of pistachio cultivars with increasing gibberellin concentrations.

Brassinosteroids, having been first found in the bee–collected pollen of rape plants (Grove et al., 1979), are the least investigated growth regulators in terms of their effects of pollen germination and growth. Extensive studies in Arabidopsis have shown that brassinosteroids are important in the regulation of reproductive growth (Kang and Guo, 2011). Hewitt et al. (1985) proposed that pollen tube elongation could depend on brassinosteroids. Brassinosteroids were reported to improve pollen germination and tube growth in *Camelia japonica* (Hewitt et al., 1985), tomato (Singh and Shono, 2003) and grapevine (Gökbayrak and Engin 2016).

Pomegranate is an andromonoecious species having both bisexual flowers and functionally male flowers on the same plant. The previous studies on pomegranates showed that *in vitro* pollen germination ratio was affected by genotypes or methods used and the medium or chemical concentration in the medium (Gozlekci and Kaynak, 2000, Melgarejo et al., 2000; Engin and Hepaksoy, 2003). This study was conducted to determine the effects of a brassinosteroid compound, 24–epibrassinolide, along with the two more commonly tested growth regulators, gibberellins and auxins, on *in vitro* pollen germination of pollen collected from functional male and bisexual flowers of pomegranate cultivars.

Materials and Methods

Both functional male and bisexual flowers of the pomegranate (*Punica granatum* L.) cultivars, ‘Antalya–14’, ‘Aşınar’ and ‘Çekirdeksiz’ were collected from the collection orchard located at the Dardanos campus of Çanakkale Onsekiz Mart University, Çanakkale, Turkey. These are cultivars bred through selection breeding from Antalya region. Flowers of different sexual morphs were hand–picked and taken to the laboratory.

Their anthers were detached slowly using a forceps and then put on a paper at room temperature of 22°C for about 12–18 hours to dehydrate some of their moisture and split so that pollen would be released. Types and concentrations of the plant growth regulators used were as follows: naphthalene acetic acid (NAA) with concentrations of 0.5, 1.0 and 2.5 ppm; gibberellic acid (GA_3) with concentrations of 25, 50, and 100 ppm; epibrassinolide (EBR, Sigma E1641) with concentrations of 0.001, 0.01, and 0.1 ppm for germination of pollen grains of three pomegranate cultivars in culture medium with 20% sucrose and 1% agar in 26±1°C under 8 hrs dark and 16 hrs daylight condition in 2014. Twenty–four hours later, the pollen grains were examined under light microscopy (Olympus RX–41 microscope at 10x magnification). Germination percentage was determined over four field views by dividing the number of germinated pollen grains by the total number of pollen grains per field of view.

The statistical analysis was performed using statistical analysis software MINITAB (Minitab Inc., ver.16 for Windows), and the significant means were compared using Tukey’s test.

Results and Discussion

Investigation on different aspects of pollen grains coming from different sources (i.e., bisexual versus. male flowers) can enable growers and breeders to better select best crossing pollinators. *In vitro* pollen germination of pomegranate flowers belonging to two different sexes were tested on plant growth regulators. Pollen collected from bisexual and functional male flowers of the cvs. ‘Antalya–14’, Aşınar’ and ‘Çekirdeksiz’ showed significant differences in response to the growth regulators added to the basal medium comprising 1% agar and 20% sucrose (Table 1).

In the bisexual flowers of cv. ‘Antalya–14’, NAA 0.5 ppm, EBR 0.1 ppm, and 25 ppm GA_3 resulted in more than 40% germination. The lowest germination was obtained in the control and GA_3 100 ppm applications (less than 10%). The promoting effects of the growth regulators were in the decreasing order of EBR, NAA, GA_3 and the control. The effects of the increasing concentrations of the growth regulators had a tendency of decreasing germination ratios in gibberellic acid and NAA treatments, but not in the epibrassinolide applications. In the functional male flowers, on the other hand, the highest germination was from the 100 ppm GA_3 application (80%), while the lowest ones were from both NAA and the control. The magnitude of the growth regulators were in the decreasing order of EBR, GA_3, NAA and the control. There were also significant differences between the types of the flowers, having greater germination ratio in the functional male flowers in the applications of 100 ppm GA_3, 0.001 and 0.01 ppm EBR, and the control. Pollen in the control group of the flower types were less than 10% in the bisexual and little over 28% in the functional male.

Table 1. Comparison of pollen sources (bisexual vs. functional male flowers) within the pomegranate cultivar for their *in vitro* pollen germination ratio (%) when treated with gibberellic acid (GA_3), naphthalene acetic acid (NAA) and 24–epibrassinolide (EBR).

Treatments ppm	Antalya–14		Aşınar		Çekirdeksiz	
	Bisexual	**Functional male**	**Bisexual**	**Functional male**	**Bisexual**	**Functional male**
GA_3 25	40.15 ABC a*	44.61 CD a	35.69 ABC a	31.53 BCD a	53.82 A a	51.38 A a
GA_3 50	25.27 C b	45.03 CD a	16.82 D b	59.24 A a	34.55 B a	34.63 CDE a
GA_3 100	9.24 D b	80.85 A a	18.63 CD b	41.44 BC a	26.36 BC a	30.45 CDEF a
NAA 0.5	48.22 A a	36.04 DE a	24.96 BCD a	39.41 BCD a	24.37 BC b	41.91 ABC a
NAA 1.0	30.06 BC a	35.21 DE a	29.36 ABCD a	33.04 BCD a	31.84 B a	24.90 EF a
NAA 2.5	26.88 C a	37.27 DE a	21.67 BCD a	35.02 BCD a	29.08 B a	28.84 DEF a
EBR 0.001	33.77 ABC b	62.26 B a	39.04 AB a	48.36 AB a	15.85 C b	47.13 AB a
EBR 0.01	39.53 ABC b	57.45 BC a	45.57 A a	26.71 CD b	29.02 B a	38.43 BCD a
EBR 0.1	42.94 AB a	50.41 BCD a	11.54 D b	41.80 BC a	36.17 B a	22.20 F b
Control	9.40 D b	28.32 E a	43.49 A a	22.25 D a	6.85 C b	22.69 EF a

*While capitals letters annotate the differences among the treatments, small letters show the differences between the pollen sources in a given treatment.

In the cv. 'Aşınar', bisexual flowers had the highest germination ratio (higher than 40%) from the EBR 0.01 ppm and the control group, followed by EBR 0.001 ppm. EBR with 0.1 ppm and GA_3 50 ppm resulted in the lowest ratio (less than 12%). EBR>control>NAA>GA_3 was the decreasing order of the hormones in inducing pollen germination. Increased concentrations of GA_3 caused pollen to germinate at a lower percentage. Pollen from the functional male flowers were most induced to germinate by the applications of GA_3 50 ppm and EBR 0.001 ppm. Control group had the lowest ratio (22%). Difference between the flower types was evident in the applications of GA_3 50 and 100 ppm and EBR 0.1 ppm, being higher in the functional male flowers.

In the cv. 'Çekirdeksiz', the highest germination was received from the GA_3 25 ppm treatment (approximately 54%) in the bisexual flowers. The lowest ratio, on the other hand, was obtained from the control group. Increasing GA_3 concentrations had a tendency to lower the germination ratio considerably. NAA effect was not dependent on the concentrations. EBR, on the contrary, induced the ratio as the concentrations were greater. In the functional male flowers, pollen germination was 51% in the GA_3 25 ppm application, being the highest. The effects of the other applications were not easily distinct. The response of the control group was similar to the effects of GA_3 100 ppm, NAA 1.0 and 2.5 ppm, and EBR 0.001 ppm. The difference between the flower types was only discernible with the NAA 3.5 ppm, EBR 0.001 ppm, and the control, being higher in the functional males.

Yang et al. (2013) found that low concentrations of benzyladenine and 2,4–dichlorophenoxy acetic acid increased germination in 'Taishanhang' pomegranate. The auxin used in this study, NAA, had also improving effects depending on the type of flower and cultivar. Although they did not find significant differences in the sucrose ratio in the basal medium, Malgarejo et al. (2000) showed that the male flowers of ME15 clone of pomegranate gave a higher germination *in vitro* than the bisexuals when low sucrose concentrations were used. In this study, functional male flowers were higher in the cvs. 'Antalya–14' and 'Çekirdeksiz', but not in the cv. 'Aşınar' when control groups were considered. This means that genotype is a factor regulating this effect. On the other hand, pollen from functional males resulted in higher germination ratios in values in most of the treatments, but in general, difference was not significant. Gözlekçi and Kaynak (2000), in support of this study, found that there was no difference in the germination ratios of the pollen derived from the two flower types in cv. 'Aşınar'. However, they observed significant differences in the other cultivars, also in consistent with this study. Derin and Eti (2001) reported that there was a significant difference in germination ratio between the two types of flowers in the cv. '33 N 26', but not in the cv. 'Hicaz'. Imani and Nazarian (2013) reported that pollen of 'Alktorshsaveh' and 'Alktorshtabrizi' pomegranate cultivars were cultured in a medium with 10% sucrose and 1.5% agar containing different plant growth regulators and found that low concentrations of NAA (50 ppm) resulted in 70% germination. On the other hand, high concentrations of GA_3 and NAA (150 ppm) gave the poorest ratios. Although not as high concentrations were used in this study, but in general, lower concentrations of both GA_3 and NAA were more effective on inducing germinations. One challenging issue with the study in hand to compare with previous studies is that the gender of the flower for which the germination ratios was investigated was not specified in most of them. Engin and Gökbayrak (2015) *in vitro* treated pollen of 'Mayhoş–8' pomegranate cultivar with GA_3, NAA and EBR, and found that pollen from the functional male flower germinated significantly higher than those from the bisexual flower (24.10% vs. 8.88%). Plant growth regulators used boosted the ratio of germination specifically in bisexual flower, 0.1 ppm EBR giving the highest one.

Both staminate and perfect flowers in the same individual, andromonoecy, have been found to occur in less than 2% of all flowering plants (Yampolsky and Yampolsky 1922). One of the theories in an attempt to explain the evolution of andromonoecy is that efficiency at donating pollen is higher in staminate flowers for any reasons of more or larger pollen, greater activity to pollinator agents or increased pollen viability (Barrett 2002). Comparison between the pollen germination of bisexual and functional male flowers of the pomegranate cultivars studied clearly showed that pollen donation from the male flowers might be of greater value, which could result in increased outcrossing in the case of cvs. 'Antalya 14' and 'Çekirdeksiz'. Pollen from the bisexual flowers of cv. 'Aşınar' can also be of greater value in selfing or pollination studies.

Conclusions

This experiment showed that germinating ability of pollen collected from the different sources in the cultivars is inherently different. Functionally male flowers generally had higher *in vitro* pollen germination than the bisexual flowers. Inducing effects of plant growth regulators in pomegranates depend on their type and concentration, and genotype and source of the pollen. Increased concentrations of gibberellic acid generally reduced germination and epibrassinolide increased germination ratio, although not in a constant manner.

References

Acar, I., Ak, B.E., Sarpkaya, K., 2013. Effects of boron and gibberellic acid on *in vitro* pollen germination of pistachio (*Pistacia vera* L.). Afr. J. Biotech. 9(32): 5126–5130.

Barrett, S.C.H., 2002. The evolution of plant sexual diversity. Nature Reviews Genetics. 3: 274–284.

Derin, K., Eti, S., 2001. Determination of pollen quality, quantity and effect of cross pollination on the fruit set and quality in the pomegranate. Turk J Agric For. 25: 169–173.

Engin, H., Gökbayrak, Z., 2015. Effect of plant growth regulators on pollen germination of 'Mayhoş–8' pomegranate cultivar. Bahçe Special Issue of Proceedings of 7th Nat'l Horticulture Congress, August 2529, 2015, Çanakkale, Turkey, 991–994 (in Turkish with English abstract).

Gadže, J., Radunić, M., Petric, I.V., Ercisli, S., 2011. In vitro pollen viability germination and pollen tube growth in some pomegranate (*Punica granatum* L.) cultivars from Croatia and Bosnia and Herzegovina. Acta Sci. Pol., Hortorum Cultus. 10(3): 297–305.

Gokbayrak, Z., Engin, H., 2016. Effect of plant growth regulators on in vitro pollen germination of grapevine cultivars. Acta Hortic. 1139: 405–408.

Gözlekçi, Ş., Kaynak, L,. 2000. Investigations on pollen production and quality in some standard pomegranate (*Punica granatum* L.) cultivars. In : Melgarejo P. (ed.), Martínez–Nicolás J.J. (ed.), Martínez–Tomé J. (ed.). Production, processing and marketing of pomegranate in the Mediterranean region: Advances in research and technology. Zaragoza: CIHEAM, p. 71–77 (Options Méditerranéennes: Série A. Séminaires Méditerranéens; n. 42).

Grove, M.D., Spencer, G.F., Rohwedder, W.K., Mandava, N.B., Worley, J.F., Warthen Jr., J. D., Steffens, G.L., Flippen–Anderson, J.L., Cook Jr. J.C., 1979. Brassinolide a plant growth promoting steroid isolated from *Brassica napus* pollen. Nature. 281: 21–217

Hewitt, F.R., Hough, T.O., Sasse, J.M., Williams, E.G., Rowon, K.S., 1985. Effect of brassinolide and other growth regulators on the germination and growth of pollen tubes of *Prunus avium* using a multiple hanging–drop assay. Aust. J. Plant Physiol. 12: 201–211.

Imani, A., Nazarian, M., 2013. Effects of boron and growth regulators on germination of *Punica granatum* pollen grains. Advanced Crop Science. 3: 268–272.

Kang, Y.Y., Guo, S.R., 2011. Role of brassinosteroids on horticultural crops. In: Brassinosteroids: A Class of Plant Hormone, Springer Science+Business Media, New York. pp. 14–192

Melgarejo, P., Martínez, J.J., Hernández, F., 2000. A study of different culture media for pomegranate (*Punica granatum* L.) pollen. In: Melgarejo P. (ed.), Martínez–Nicolás J.J. (ed.), Martínez–Tomé J. (ed.). Production, processing and marketing of pomegranate in the Mediterranean region: Advances in research and technology. Zaragoza: CIHEAM, pp. 63–69 (Options Méditerranéennes: Série A. Séminaires Méditerranéens; n. 42)

Singh, I., Shono, M., 2003. Effect of 24–epibrassinolide on pollen viability during heat–stress in tomato. Indian J. Exp. Bot. 41: 174–176.

Tosun, F., Koyuncu, F., 2007. Effects of some chemical treatments on pollen germination and pollen tube growth in sweet cherries (*Prunus avium* L.). Akdeniz Univ. Journal of Faculty of Agriculture. 20: 219–224.

Vitagliana, C.R., Viti, R., 1989. Effects of some growth substances on pollen germination and tube growth in different stone fruits. Acta Hortic. 239: 379–382.

Viti, R., Bartolini, S., Vitagliano, C., 1990. Growth regulators on pollen germination in olive. Acta Hort. 286: 227–230.

Yampolsky, C., Yampolsky, H., 1922. Distribution of sex forms in phanerogamic flora. Bibliotheca Genetica. 3: 1–62.

Yang, S., Yuan, Z., Li, Y., Li, Q., Yin, Y., Feng, L., Zhao, X., 2013. Biological characteristics of pollen germination of 'Taishanhong' pomegranate. Scientia Silvae Sinicae. 49:48–53.