

## Assessment of pollen quality and quantity in white and black Turkish *Myrtus communis* L. accessions, through *in vitro* pollen germination under varied boric acid concentrations

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Received: 29.08.2023; Revised: 04.10.2023; Acceptance: 06.10.2023

DOI: 10.53552/ijmfmap.9.2.2023.167-176

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### ABSTRACT

This study aimed to evaluate the pollen properties, pollen viability and germination with suitable germination medium and amount of pollen production in 6 Turkish myrtle genotypes. The genotypes were evaluated as their origin and fruit colours in terms of 3 different origins (Erdeмли, Karaisali and Tarsus) and 2 fruit colours (black and white). Results showed that, pollen properties were affected from fruit colours and black genotypes generally showed higher pollen properties. The amount of pollen production of one flower was very high ranging from 2.5 to 4.5 million. Pollen viability and germination rates showed sufficient results for successful pollination in Turkish myrtle genotypes. Among pollen germination media, the best pollen germination rate was obtained from 50 ppm boric acid. Consequently, this study identified the pollen performance of white and black Turkish myrtle genotypes and suggested an optimal pollen germination medium for *Myrtus communis* which can be used in the future breeding programmes.

**Keywords:** Anther, flower, Myrtaceae, pollen biology, pollination

### INTRODUCTION

Myrtle (*Myrtus communis* L.) belongs to the family Myrtaceae and is one of the most significant species widely spread in the Mediterranean region, the Middle East and warmer regions of North America and Australia. In Turkey, it has been intensively grown naturally in the Mediterranean region especially near natural pine forests, particularly in the Taurus Mountains and riversides. The species are growing wild in warm and temperate regions of the Mediterranean basin, where it is well known for its medicinal and aromatic properties (Mulas *et al.*, 1999; Yildirim *et al.*, 2013). Essential oil from the leaves is used in the perfume and food industries (Lawrence, 1994; Boelens and Jimenez, 1992), while both leaves and berries are used to produce typical liqueurs (Mulas *et al.*, 1999). Due to the success of myrtle liqueur and the number of processing industries, the demand for raw material has increased (Mulas and Fadda, 2004)

Myrtle has several genotypes with yellowish-white or bluish-black coloured fruits (San *et al.*, 2015; Simsek *et al.*, 2020). Recent studies of myrtle

have focused on the health functions of aromatic and medicinal plants, which have antioxidant, antimicrobial and mutagen properties due to the dietary intake of antioxidant compounds (Duh *et al.*, 1999; Yildirim *et al.*, 2013). Myrtle oil has a very extensive consumption as food, cosmetics, medicine, perfumery etc. (Jamoussi *et al.*, 2005; Mechchate *et al.*, 2022). In recent years as it has been understood as an important fruit, it gained importance and started to take place in markets and in herbalists (Montoro *et al.*, 2006; Alim, 2020). Due to high food value and wide usage of myrtle fruits and leaves, its production has been increased. Whether there is not a closed myrtle orchard in Türkiye, plants were started to plant as hedge plant in order to provide the demand of customers.

Myrtle flowers need pollination to set fruits and a successful pollination depends on quality of flowers especially in terms of pollen quality. Myrtle fruits has lots of seeds (Mulas and Fadda, 2004). Whether this seed existed disturbs customers, the studies for decreasing seed number caused excessive fruit drops (Gonzalez-Varo *et al.*, 2009, Alim, 2020). This shows that, seed formation after

successful pollination and fertilization is essential for adequate fruit set in myrtle.

Myrtle buds develop from the leaf axillae on young shoots as single flowers (Mulas and Fadda, 2004). It has a 1-2,5 cm green or red peduncle generally linked with fruit colour. Myrtle flowers are hermaphroditic with both stamens and pistilare at the same flower (Fig. 1a). Flowers comprise of 5 green sepals, 5 white or pink petals, about 100-150 stamens with long filaments and a little anther. Pollens are triangular in shape and hastric orporate apertures with a diameter about 8-15mm (Fig. 1b). The flower has a single pistil at the middle of the flower. It is embedded into the receptacle showing an inferior ovary type with 3 syncarpous carpels with axile placentation of about 25-50 ovules (Mulas and Fadda, 2004). Flowers secrete lots of nectar at the bottom of the anthers and originally near from the apex of the anther in order to attract pollinator insects for a better pollination (Ciccarelli *et al.*, 2008).

Recent studies have been showed that, myrtle pollen viability is very high immediately after anthesis. Along with the end of the first day, pollen viability declines and 36 hours after anthesis almost all grains become unviable. At the same time, while pollen viability remains high for several hours at high temperature and dry conditions, it rapidly decreases at high humidity (Aronne, 1999). So, myrtle pollen should be transferred to the stigma as soon as possible by pollinator insects which indicates the importance of pollen limitation and pollen viability on more time. Although myrtle is a self-compatible species and needs pollinator to increase fruit set, fruit size and quality increases with cross pollination showing that the pollen quality has a great importance for myrtle fruit formation (Aizen and Harder, 2007; Gonzalez-Varo *et al.*, 2009).

Considering the effects of pollen importance in myrtle flowers, this study aims to evaluate the pollen quality in terms of pollen viability and pollen germination rates, suitable *in vitro* pollen germination medium and *in vitro* pollen tube growth in 3 black and 3 white Turkish myrtle genotypes. At the same time, the amount of pollen production was also determined for relevant genotypes.

## MATERIALS AND METHODS

This study was conducted in 2023 flowering season of 6 Turkish myrtle genotypes. The genotypes were selected for their superior qualities from Mediterranean region of Türkiye (Simsek *et al.*, 2020). The selected genotypes were from 3 different regions of Türkiye (Erdemli, Karaisalı and Tarsus) due to their fruit colours (white and black) (Table 1). These selected genotypes were planted in research field of Cukurova University in 2020 at 3x2 m spacing. Plants were 3 years old, drip irrigated and all necessary cultural practices were followed uniformly in the research field.

In this study the amount of pollen production and pollen homogeneity, *in vitro* pollen viability and germination rates, suitable *in vitro* pollen germination medium and *in vitro* pollen tube growth rate parameters were evaluated. For this purpose, at least 100 unopened flowers were collected one day before anthesis from 5 trees of each genotype and immediately taken to the Cytology Laboratory in Cukurova University.

### Amount of pollen production and pollen homogeneity

For determining amount of pollen production and homogeneity tests, 30 flowers were separated to 3 groups and anthers of each group (10 flowers for each) were taken to plastic containers. Prepared samples were left to dry for at least 15 days. Pollen production tests were made by hemacytometric method and counting slides were prepared according to Eti (1990) with 4 ml of sterile water (Fig. 2a). Then, the samples were analysed with Olympus BX51 light microscope and micrographs were taken with DP72 camera with a specified area on the micrograph (Fig. 2b). Following preparation, the quantity of pollen within the specified area was tallied, and subsequent calculations were performed, incorporating a modification based on Eti (1990). While calculation, the counting place volume was found with multiplication of the created area and depth of hemacytometric slide. The amount of pollen at calculated volume was then rated to whole container volume to find "amount of pollen for 10 flowers". Then the "pollen number per flower" was found by dividing each data to 10. At least 12 areas were counted for each replication and the average data were used for calculation.

The “normally developed pollen ratio” was also determined in each micrograph by counting and rating normally developed pollen to total pollen in created volumes according to Anvari (1977).

### **Invitropollen viability rate**

In order to obtain pollen for viability tests, anthers from 6 genotypes of myrtle were collected randomly before anthesis. Anthers of the collected flowers were separated and placed in room temperature until dehiscence for about one night (Mulas and Fadda, 2004). After pollen dehiscence, pollen viability rates were determined using 1% TTC test (2,3,5-Triphenyltetrazoliumchlorid). TTC was prepared and evaluated according to Norton (1966). For each genotype 3 slide replication were prepared and at least 100 pollen grains were counted from each replication. Pollen counting was made by Olympus BX51 microscope. While counting, red pollens were considered as “viable”, light pinkies as “semi viable” and uncoloured pollens as “non-viable” (Fig. 3a). Then the pollen viability rate was calculated according to Norton (1966) as the ratio of “sum of total viable and half of semi viable pollens” to “total pollen number”.

### **Suitable invitropollen germination medium and invitropollen germination rate**

In order to determine pollen germination data, same pollens were used that was prepared for pollen viability tests. The suitable *in vitro* pollen germination tests were all performed in a basic media, which consisted of 1% agar, 100 ml distilled water and 15% sucrose (Mulas and Fadda, 2004) with single factor experiment of boric acid ( $H_3BO_3$ ) in concentration of 0, 50 and 100 ppm.

Media and germination petri dishes were prepared according to Karabiyik and Eti (2016). Pollen germination ratio for each medium was determined after 24 hours. The pollen grains were considered to be germinated when the pollen tube length was greater than the diameter of the pollen grain (Fig. 3b). At least 100 pollen grains were counted for each replication.

The *in vitro* pollen tube growth rate was also determined after 2, 4 and 10 hours of preparation in order to set out the difference at emergence and elongation of pollen tubes for each media and genotype. In order to measure pollen tube length

in specified hours, little segments were taken from prepared media between slide and cover glass and immediately taken to  $-20^{\circ}C$  to stop growth and fix the media samples filled with pollen. Experiments were prepared as 3 replications for pollen tube measuring tests. The *in vitro* pollen tube growth was determined by measuring pollen tube length with Olympus BX51 microscope equipped with a DP72 digital camera (Fig. 3c). At least 30 pollen tubes were measured for each media, each hour and each replication. By this way, the fastest pollen germinating media could be determined for myrtle genotypes.

### **Statistical analysis**

All data analysis was performed using JMP13 statistical software. The effects of the treatments were analysed using one way anova analysis of variance. A P value of  $<0,05$  was considered to be significant. The statistical analysis was conducted in terms of the origins and fruit colour of genotypes. By this way, 2 factorial randomised design has been used for analysing pollen production, normally developed pollen and pollen viability data. In the other part of our experiment, the pollen germination rate was analysed by 3 factorial randomised design as genotype origin, colour and boron concentrations. Percentages were analysed after arc-sin transformation.

## **RESULTS AND DISCUSSION**

### **Amount of pollen production and normally developed pollen rate**

Amount of pollen production for 6 Turkish myrtle genotypes were given in Table 2 in terms of origins and fruit colour of the genotypes. The table shows that amount of pollen production in one flower significantly influenced by fruit colour and origin x colour interaction while differences between genotypes did not find to be important. The pollen production in each flower was very high ranging between 2657493 (Karaisali-White\_KB) and 4885013 (Karaisali-Black\_KS) pollens per flower and the average pollen production was definitely higher in black genotypes (3843840) than white genotypes (3056213). In terms of genotype origin averages, the highest pollen production was detected in Karaisali genotypes (3

**Table1:Originsandfruitcoloursofusedgenotypes**

Genotypename	Genotypecode	Origin	FruitColour
Erdemlibeyazi	EB	Erdemli/Mersin	Whitefruited
Erdemlisiyahi	ES	Erdemli/Mersin	Blackfruited
Karaisalibeyazi	KB	Karaisali/Adana	Whitefruited
Karaisalisiyahi	KS	Karaisali/Adana	Blackfruited
Tarsusbeyazi	TB	Tarsus/Mersin	Whitefruited
Tarsussiyahi	TS	Tarsus/Mersin	Blackfruited

**Table2:Amountofpollenproductionperflowerin6Turkishmyrtlegenotypesintermsoftheir origins and fruit colours (pollen/anther)**

Origins	Fruit Colours		OriginsAverage
	White	Black	
Erdemli	3295146b <sup>1</sup>	3 412 266b	3 353 706
Karaisali	2657493 b	4885013 a	3771253
Tarsus	3216000 b	3234240 b	3225120
<b>ColoursAverage</b>	3056213 B	3843840 A	
LSD <sub>origin</sub> ***:N.S. <sup>2</sup>	LSD <sub>colour</sub> **: 586 814,2	LSD <sub>orixcol</sub> ***:1 016 392,0	

<sup>1</sup>Differencesbetweenaveragesshowedbydifferentlettersarestatisticallysignificant  
N.S.meansnot-significant; \*\*meansp<0,01; \*\*\*meansp<0,001.

**Table3:Normallydevelopedpollenratiosin6Turkishmyrtlegenotypesintermsoftheirorigins and fruit colours (%)<sup>1</sup>**

Origins	FruitColours		OriginsAverage
	White	Black	
Erdemli	93.17	93.97	93.57A <sup>2</sup>
Karaisali	89.35	88.02	88.68B
Tarsus	93.95	93.75	93.85A
<b>ColoursAverage</b>	92.16	91.91	
LSD <sub>origin</sub> ***:1,985	LSD <sub>colour</sub> :N.S.	LSD <sub>orixcol</sub> :N.S.	

<sup>1</sup>Statisticalanalysisweremadeafterarc-sintransformation.

<sup>2</sup>Differencesbetweenaveragesshowedbydifferentlettersarestatisticallysignificant  
N.S.meansnot-significant; \*\*\*meansp<0,001.

**Table4:Pollen viability levels in 6 Turkish myrtle genotypes in terms of their origins and fruit colours (%)<sup>1</sup>**

Origins	FruitColours		OriginsAverage
	White	Black	
Erdemli	42.84 cd <sup>2</sup>	85.46a	64.15A
Karaisali	51.99 b	35.11d	43.55B
Tarsus	50.30 bc	44.63bc	47.47C
<b>ColoursAverage</b>	48.38 B	55.07A	

LSD<sub>origin</sub> \*\*\*:3,605    LSD<sub>colour</sub> \*\*:2,943    LSD<sub>orixcol</sub> \*\*\*:5,098

<sup>1</sup>Statisticalanalysisweremadeafterarc-sintransformation.

<sup>2</sup>Differencesbetweenaveragesshowedbydifferentlettersarestatisticallysignificant  
N.S.meansnot-significant; \*\*meansp<0,01; \*\*\*meansp<0,001.

**Table5:Pollen germination levels in 6 Turkish myrtle genotypes in terms of their origins, fruit colours and Boron concentrations (%)<sup>1</sup>**

Origins	Boron Concent.	FruitColours		Originx Boron	Origins Average
		White	Black		
Erdemli	0ppm	26,20	46,34	36,27	41,58
	50ppm	27,99	63,49	41,58	
	100 ppm	19,68	65,79	46,89	
<b>Originx Colour</b>		24,62b <sup>2</sup>	58,54a		
Karaisali	0ppm	31,00	68,07	49,53	42,53
	50ppm	37,45	37,27	37,36	
	100 ppm	26,04	55,37	40,71	
<b>Originx Colour</b>		31,50b	53,57a		
Tarsus	0ppm	47,17	43,57	45,37	51,34
	50ppm	51,15	67,14	59,15	
	100 ppm	50,54	48,49	49,52	
<b>Originx Colour</b>		49,62a	53,07a		
<b>ColoursAverage</b>		35,25B	55,06A		
LSD <sub>origin</sub> :N.S.	LSD <sub>colour</sub> ***:4,919	LSD <sub>boron</sub> : N.S.	LSD <sub>ori x bor</sub> : N.S.	LSD <sub>orixcol</sub> ***:8,521	
LSD <sub>orixcolxbor</sub> : N.S.					

<sup>1</sup>Statisticalanalysisweremadeafterarc-sintransformation.

<sup>2</sup>Differencesbetweenaveragesshowedbydifferentlettersarestatisticallysignificant  
N.S.meansnot-significant;\*\*\*meansp<0,001.

771253),followedbyErdemli(3535706)and Tarsus(3225120)genotypes.

Pollenproductionisanimportantdatafor seededcultivarsasitguaranteesthepollenmeeting probabilitywiththestigma.Inthisstudy,amount ofpollenproductioninonemyrtleflowerwasvery highatabout2.5millionsand4.5millions.However, myrtleflowershavealotofanthers between120-150(MulasandFadda,2004)andthis means each anther has at about 15 000 – 30 000 pollens.Tothebestofourknowledge,thereisnot anydataforamountofpollenproductioninmyrtle flowersandthisreportisthefirstforpollen productionfor*Myrtuscommunis*.However,thedata wascoherentwithotherspecieslikecitrus (KarabiyikandEti,2019),carob(Eti,1990), strawberry(Karabiyik<sup>etal.</sup>,2016),pecan (KarabiyikandEti,2018),watermelon(Adiguzel <sup>etal.</sup>,2022),loquat(KarabiyikandEti,2015)etc. intermsofamountofpollenproducedinoneanther.

Nevertheless, pollen production for myrtle genotypes should be higher than most of the species.Because,Beardsell<sup>etal.</sup>(1989)havebeen stated that members of Myrtaceae has secretes underanthersofthestamenscalledantherglands

thathelpsflowerstoattractpollinatorinsects, especiallythebees.Theauthorshavebeenreported thatthissecretemixeswithpollenwhichactsasa foodsourceforinsects(Ciccarelli<sup>etal.</sup>,2008).This structureofflowerscauseslotsofpollenloses,so thehighpollenproductionrolesoutthesehandicap. Normally developed pollen ratios were given in Table 3. The data showedthat normally developedpollenwhichshowspollenhomogeneity level is generally high and only origin of the genotypeaffectsnormallydevelopedpollenratio. The highest ratio was obtained from Erdemli (93.57%) and Tarsus (93.85%) genotypes while Karaisali genotypes (88.68%) had the lowest homogeneity level. Fruit colour did not differ significantly and white genotypes had an average of 92.16% while black genotypes has an average of 91.91% pollen homogeneity level.

Besidesthe high pollen productionof a genotype, it is important to have a genotype high normally developed pollen ratio. Anvari (1977) havebeenstatedthattheundevelopedorabnormal pollens,whichshowsdiversionsfromnormalshape andsize,areunlikelytogerminate.So,ifthenormal rateofpollenratioapproachto100%,the

Pollen quality and quantity in Myrtle



Fig. 1: Flower (a) and pollen (b) of myrtle

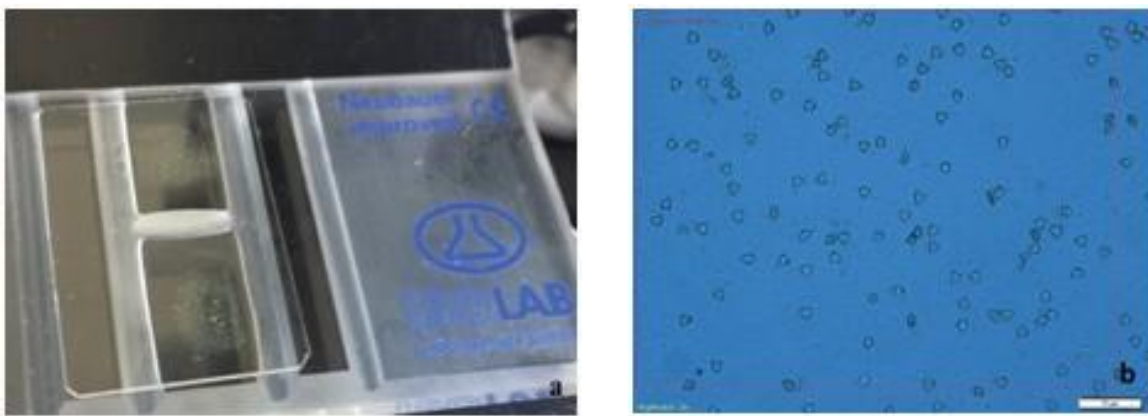


Fig. 2: The hemacytometric slide (a) and the pollen counting area (b)

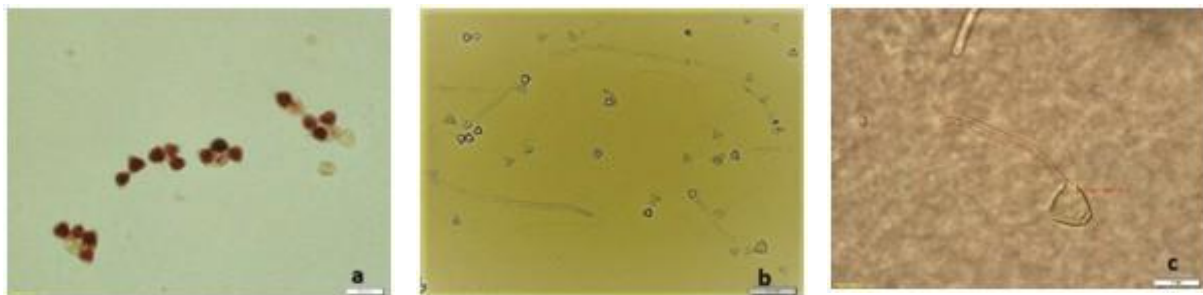


Fig.3: a. pollen viability test, b. germinated pollens, c. Pollen tube elongation

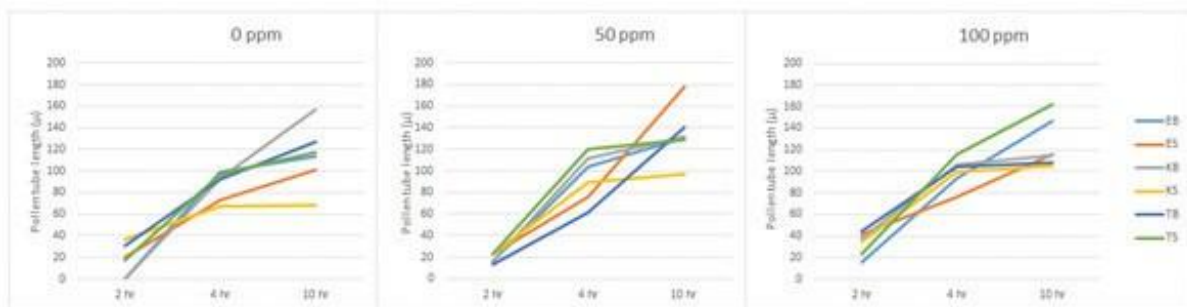


Fig.4: Pollen tube growth rate of Turkish myrtle accessions in different *in vitro* pollination media.

pollination potential of genotype will increase in the same rate. The results showed that myrtle pollens have adequate pollen homogeneity and pollen production level for successful pollination.

### ***In vitro* pollen viability rate**

*In vitro* pollen viability have been detected by TTC method and the results were shown in Table 4. The statistical analysis showed that origin, colour and origin x colour interactions significantly affected pollen viability rate of used myrtle genotypes. Pollen viability rate was highest in Erdemli originated genotypes with 64.15% while it was 47.47% in Tarsus and 43.55% in Karaisali. In terms of fruit colours, average of black cultivar showed better results (55.07%) than white genotypes (48.38%). The origin x colour interaction data had a wide range as much as 35.11% (Karaisali-Black\_KS) and 85.46% (Erdemli-Black\_ES).

As Mulas and Fadda (2004) have been reported, pollen viability rate was not differed between freshly opened flowers and dried flowers and the pollen viability rates of 10 myrtle genotypes were higher than our results that differed between 85% and 95%. It is thought that this difference is originated from differences in genotype and ecological conditions (Eti, 1991).

### ***In vitro* pollen germination rates and suitable pollen germination media**

Table 4 shows *in vitro* pollen germination levels of 6 Turkish myrtle genotypes in terms of their origin and fruit colours as well as different germination media. In the study, different concentrations of boric acid ( $HBO_3$ ) were retested for optimization of *in vitro* pollen germination medium. The results showed that boric acid concentration has no importance on pollen germination ratio while fruit colour and origin x colour interaction (genotype effect) showed statistically important values.

In terms of fruit colours, pollen germination levels were higher in black genotypes (55.06%) than white genotypes (35.25%), like pollen production and pollen viability levels. Origin x colour interaction that shows the germination levels of each genotype were highest in Erdemli-Black (ES) which was followed by Karaisali-black (KS) and Tarsus-black (TS) and the lowest from Erdemli-white (EB).

In general, pollen germination level gives the best results for the pollen quality of a genotype. Although genotypes should have a good pollen production and pollen viability percentage; pollen germination, pollen tube emergence and pollen tube growth has such a degree importance for pollination and especially for fertilization level. In a recent study, Mulas and Fadda (2004) tested 10 myrtle genotypes in terms of pollen germination levels with 10, 15 and 20% sucrose concentrations and found the rates of 6 genotypes as 70 to 85%; other 3 genotypes differing from 25 to 40% and one genotype with 0.7%. Authors reported the best media as 10 and 15% sucrose concentrations for most of the genotypes. The 15% sucrose concentration that was used in this study was suitable for myrtle pollen germination. Despite the statistical analysis has no significant importance, a 50 ppm Boric acid addition to germination media could increase pollen germination rate for most of the genotypes.

Pollen germination assays frequently require optimisation as well as being time consuming and difficult to reproduce (Rathod *et al.*, 2018). The germination media should imitate stigma surface for the truest data. It has been reported that, boric acid content in the pollen is insufficient (O'Kelley, 1957) and Boron in the stigma and style is required to compensate for this; thus a certain amount of boric acid needs to be added to *in vitro* cultures (Johri and Vasil, 1961; Luo *et al.*, 2020). There was not any test for additional substances for *Myrtus communis*. In general, 10 ppm (0.001%) or higher concentrations of Boric acid is toxic to plant growth while pollen grains can tolerate concentrations up to 1 200 ppm (0.12%) and optimum results were obtained between 10 and 150 ppm concentrations for pollen germination for most of the species (Johri and Vassil, 1961).

In this study, the results of viability and germination rates were in parallel except from Karaisali-black (KS). The chemical TTC (Tetrazolium) forms a red coloured compound formazan by H transfer reactions catalysed by the enzyme dehydrogenases (Norton, 1966). This results shows that, only TTC test might not give the real performance for some myrtle genotypes due to its possible insufficient reaction capacity with  $H^+$  ions. So, different viability tests

accomplished with *in vitro* pollen germination could be used for better results in future studies.

Pollen tube growth rate was also evaluated in this study and results were given in Fig. 4. The pollen tube growth rate was faster in 50 ppm boric acid concentrations which means pollen tubes will emerge and grow faster in a boron fertilized genotype. In 0 ppm Boron (Control) especially KB and EB did not emerge any pollen tubes in first 2 hours while KS and ES could not show their real performance of pollen tube acceleration. At the same time, 100 ppm Boric acid also increases the first emergence and formed faster pollen tubes.

It is known that boron accelerates pollen tube by its promoting absorption of sugars and their metabolism by forming sugar-borate complexes, increased oxygen uptake and is involved in the synthesis of pectic materials required for the wall of the actively elongating pollen tube (Johri and Vasil, 1961). As pollen did not have sufficient boron content and stigma and style will compensate boron requirement, addition of boric acid to the germination medium simulates this situation and shows the real potential of performance of pollen tube elongation (Johri and Vasil, 1961; Jayaprakash *et al.*, 2018; Luo *et al.*, 2020). When Table 5 and Figure 4 were evaluated together boric acid was thought to be accelerated the first pollen tube emergence and pollen tube elongation ratio but not have any effect on pollen germination ratio.

## CONCLUSION

Pollen quality and quantity is vital for understanding fertility and incompatibility, pollen-pistil interaction, breeding, crop improvement and seed industry. For successful pollination, the insight knowledge of pollen biology including pollen viability, germination and amount of pollen production is required for reasonable approaches to increase productivity. This study evaluates amount of pollen production, normally developed pollen ratio, pollen viability and germination levels with suitable pollen germination medium as well as pollen germination rates of 6 Turkish myrtle genotypes in terms of their origins and fruit colours. In *Myrtus communis*, amount of pollen production per flower was found to be very high as much as 2.5-4.5 million. The high pollen production is thought to be related with anther secret that will

cause pollen losses. Normally developed pollen ratio was also high and sufficient for successful pollination. The pollen viability and germination rates were sufficient for most of the genotypes and pollen tube elongation was faster in the media containing 50 ppm boric acid with 15% sucrose and 1% agar solution which is thought to be the best media for *Myrtus communis* pollen germination tests. In general, all pollen properties were higher in black genotypes than the white genotypes.

In future studies, pollination capacity of white cultivars should be experienced for its possible stimulatory effect because of their lower pollen characteristics. So, a pollination study should be planned for potential seed number decreasing effect of white genotypes.

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