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A study of different culture media for pomegranate (*Punica granatum* L.) pollen

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SUMMARY – We test different culture media and environmental conditions in an attempt to improve our knowledge of the most suitable culture media for studying the germinative capacity of pomegranate pollen. Pollen belonging to the clone ME15, a Mollar type, was used because of its regular and abundant flowering and fruit production. We observed that the incorporation of the nutrients Ca^{+2} and B^{+3} (0.2 ml/100 ml) in the medium increased the germination percentage. It was also seen that 1% agar gave better results than 1.5%. High percentages of saccharose (10-20%) did not improve germination, although 1-3% did produce notable differences. Similar improvement was observed in an experiment using 3-5-10% saccharose. We therefore conclude that the optimum saccharose concentration is between 10 and 20%, with levels below 10% producing low levels of pollen germination. We also studied the effect of two temperatures (15 and 25°C) and two incubation times (24 and 48 hours), and the possible influence of the sex of the flowers from which we took the pollen (hermaphrodite and male).

Key words: Culture medium, viability, germination potential, saccharose, agar.

RESUME – *"Etude de différents milieux de culture pour le pollen de grenade (*Punica granatum *L.)". Nous avons testé différents milieux de culture et conditions environnementales pour essayer d'améliorer notre connaissance des milieux de culture les plus appropriés pour étudier la capacité germinative du pollen de grenadier. Le pollen appartenant au clone ME15, de type Mollar, a été utilisé en raison de sa floraison régulière et abondante et de sa production fruitière. Nous avons observé que l'incorporation des nutriments Ca^{+2} et B^{+3} (0,2 ml/100 ml) dans le milieu augmentait le pourcentage de germination. On a vu également que 1% d'Agar donnait de meilleurs résultats que 1,5%. De hauts pourcentages de saccharose (10-20%) n'ont pas amélioré la germination, bien que 1-3% aient produit des différences notables. Une amélioration semblable a été observée dans une expérience en utilisant 3-5-10% de saccharose. Notre conclusion est donc que la concentration optimale de saccharose se trouve entre 10 et 20%, avec des niveaux en dessous de 10% produisant de faibles niveaux de germination du pollen. Nous avons également étudié l'effet de deux températures (15 et 25°C) et de deux temps d'incubation (24 et 48 heures), et l'influence possible du sexe des fleurs à partir desquelles nous avons prélevé le pollen (hermaphrodite et mâle).*

Mots-clés : *Milieu de culture, viabilité, potentiel de germination, saccharose, Agar.*

Introduction

In vitro germination experiments with pollen in sugared media have been much used in numerous species, with two aims: (i) evaluation of germinative capacity; and (ii) determination of the influence of abnormal conditions, on pollen germination and the subsequent growth of the pollen tube.

The saccharose concentration has a clear influence on the germination of pomegranate pollen in artificial media (Sharma and Gaur, 1982). Sharma and Gaur experimented with culture media for pollen, with saccharose concentrations which varied between 0% and 20%, and showed that for the majority of varieties used, the highest germination levels were obtained with saccharose concentrations of between 15% and 20%. Other authors (Josan *et al.*, 1980) used saccharose concentrations of around 20%. In these studies, the incubation time for the media was 24 and 48 hours.

For pollen cultivation of some species there are established media; however, these have not been studied much in the case of pomegranates, and this work therefore intends to contribute to the improvement of our knowledge on this subject.

We will carry out three experiments, which will study not only the concentrations of the different components of the culture media, but also factors such as sex of the flower, temperature and time.

Materials and methods

The vegetable matter being studied is pomegranate pollen from the ME15 clone described by Melgarejo (1993). This is to be found on the estate of the Escuela Politécnica Superior de Orihuela (Miguel Hernández University), obtained using plant propagation, and planted in a 4×3 m layout in March 1992. The estate is in the municipal district of Orihuela (Alicante), and is watered by drip irrigation. The trees are goblet-trained.

The soil is clay loam. Rainfall is low - around 300 mm/year - and concentrated in spring and autumn. The average annual temperature is 19°C, with mild winters and hot summers, classified as subtropical in Papadakis' climatic classification (MAPA, 1986). The agroclimatic area fulfils the requirements for cultivation.

The pollen was obtained from the ME15 clone indicated above, from newly opened flowers, by shaking the anthers over Petri dishes with the help of a No. 0 brush. Two brushes were used, one for pollen from "hermaphrodite" flowers and the other for pollen from "male" flowers, thus ensuring that the two did not mix. The Petri dishes were closed and sealed for transportation to the laboratory, where they would be used to carry out the following culture media experiments.

Three tests were carried out on three different dates.

The first was carried out on 6 May 1996, to study the following factors and levels:

(i) Saccharose: we experimented with the following percentages: 10, 12.5, 15, 17.5, 20%.

(ii) Nutrients: with or without the addition of 22 mg $Cl_2Ca.2H_2O$/100 g and 2 mg boric acid/100 g of medium.

(iii) Temperature: tests were carried out in cold storage at 25°C, and at laboratory room temperature which was around 27°C.

(iv) Time: controls were carried out at 24 and 48 hours.

The *germination percentage* was the variable measured.

The second test was carried out on 5 June 1996; in this case a factorial design of 2^6 was used, in which the *germination percentage* was again the response variable or dependent variable. The design scheme was as shown in Table 1.

Table 1. Design scheme of second test

Factors	Low level	High level
Saccharose	1 g/100 ml	3 g/100 ml
Agar	0.6 g/100 ml	0.8 g/100 ml
Nutrients	0	0.2 ml/100 ml
Sex	Male	Hermaphrodite
Temperature	15°C	28°C
Time	24 hours	48 hours

When nutrients were used, the concentration was 0.183 ml of Cl_2Ca + 0.016 ml boric acid.

The third test was carried out on 6 June 1997; for this test a factorial design of 3×2^5 was used, in

which the response variable was the germination percentage, as in the previous cases. The design scheme was as shown in Table 2.

Table 2. Design scheme of third test

Factors	Low level	High level	Medium level
Saccharose	3 g/100 ml	10 g/100 ml	5 g/100 ml
Agar	1 g/100 ml	1.5 g/100 ml	
Nutrients	0	24 mg/100 ml	
Sex	Male	Hermaphrodite	
Temperature	15°C	28°C	
Time	24 hours	48 hours	

When nutrients were used, the concentration was 22 mg of Cl_2Ca + 2 mg boric acid.

In all three cases, the pollen used was from the same variety, ME15 (a clone belonging to the Mollar de Elche variety); this is a good quality productive variety.

We applied Bliss transformation to the results obtained from the experiments, and with these transformed results we carried out a variance analysis as well as the LSD multiple range test.

Collection and preparation of pollen

The pollen was collected from the ME15 clone as indicated above.

Study of the germination potential

Culture medium

Normally the culture medium is composed of saccharose, agar and distilled water. However, it has been shown that the germination percentage can be improved by some nutrients, notably calcium and boron (Dhar, 1983), and these are therefore the most commonly used if any nutrients are applied. With these experiments we have tried to clarify the true influence of these nutrients.

The media concentrations and the conditions during the experiments are as set out in the design schemes in each of the three tests.

The saccharose has a double role, acting as both an osmotic regulator (Visser, 1955; Vasil, 1964) and as a source of metabolic energy (O'Kelly, 1955).

The agar concentration, which is generally not given much importance, nevertheless appears to be fundamental in providing the necessary conditions for good hydration of the pollen and the growth of the pollen tube (Luza and Polito, 1985; Luza, 1987).

Preparation of the culture medium

A little distilled water and the nutrients are poured into a flask of precipitate, and the mixture is heated in a magnetoagitator. The saccharose and agar are then added slowly, until fully dissolved. The solution is then poured into a Erlenmeyer flask, which is plugged with cotton wool and covered with aluminium foil, then kept in a freezer for later use. Before each use, it is sterilised in an autoclave at 120°C for 15 minutes.

Sowing and counting of pollen

The sterilised culture medium is poured in a thin layer on the Petri dishes, which are suitably labelled. This process is carried out in the laminar flow chamber to avoid contamination. Once it has

solidified, the pollen is sown using a brush to distribute it as uniformly as possible on the dish. This uniformity is important to avoid the influence of "mass effect", as agglomeration of pollen grains results in higher pollen germination (Giulivo and Ramina, 1974).

The dishes are then put in cold storage at the assigned temperature for the experiment, and with light. The culture duration was 24 and 48 hours.

We consider pollen grains germinated when the length of the pollen tube is equal to or greater than the diameter of the pollen grain. The number of pollen grains counted per dish was approximately 1000.

Results and discussion

The results are presented in Tables 3, 4 and 5, showing the percentage of germinated grains in relation to the total number of grains counted.

Table 3. Pollen germination percentage with different saccharose concentrations (date: 6 May, 1996)

Saccharose (%)	Nutrients†	Temperature 25°C		Temperature 27°C	
		24 h	48 h	24 h	48 h
10	WN	11.04	12.10	14.24	15.90
	NN	1.08	1.64	1.84	2.76
12.5	WN	8.20	9.80	7.60	8.70
	NN	5.94	7.08	1.80	2.52
15	WN	6.80	7.92	10.60	15.16
	NN	2.48	3.56	2.68	3.90
17.5	WN	10.36	10.92	6.80	8.01
	NN	3.68	4.20	5.68	6.10
20	WN	8.72	9.01	6.80	8.20
	NN	3.00	3.44	4.83	5.10

†WN: with nutrients; NN: no nutrients.

On analysing the variance of the results (Table 3), highly significant differences are seen (N.S.: 1%) for the nutrient factors, i.e. the incorporation of calcium and boron nutrients has a very clear positive effect on the pollen germination percentage. This corresponds with findings of some other authors. Kwack (1965) studied the result of adding Ca^{+2} ions to the medium in 46 species, finding a favourable effect on pollen germination and pollen tube growth in all cases. Similarly, the addition of boron in the form of boric acid generally has a positive effect, as indicated by Asif and Al-Tahir (1983) in relation to the germination and pollen tube growth of date palm pollen, which are helped continuously on increasing the concentration of boric acid from 0 to 100 ppm. On other occasions, the joint addition of Ca^{+2} and boric acid has a strong effect on date palm pollen (Dhar, 1983).

The saccharose factor, with a 95% confidence level, does not show significant differences with different concentrations. A 17.5% saccharose level gives slightly higher germination percentages, but not significantly so.

The temperature factor does not produce significant differences, although at 27°C the germination percentage is slightly higher. This lack of significance is easily explained, as the temperatures were very similar.

There are not statistically significant differences in the incubation time, although we should state that after 48 h of incubation the germination percentage is considerably higher than after 24 h.

Table 4. Pollen germination percentage with natural concentrations of *in vitro* tissue culture (date: 5 June, 1996)

Pollen source and culture conditions†	Culture medium				
	Presence of nutrients††	Saccharose (3%)		Saccharose (1%)	
		Agar 0.8%	Agar 0.6%	Agar 0.8%	Agar 0.6%
M, 15°C, 24 hours	WN	3.06	3.25	3.30	2.60
M, 15°C, 24 hours	NN	1.83	6.25	3.10	2.13
H, 15°C, 24 hours	WN	3.63	2.75	2.70	1.89
H, 15°C, 24 hours	NN	3.60	2.50	0.86	2.00
M, 28°C, 24 hours	WN	4.50	5.80	3.60	4.30
M, 28°C, 24 hours	NN	5.90	4.15	3.30	2.91
H, 28°C, 24 hours	WN	5.20	4.16	3.30	3.57
H, 28°C, 24 hours	NN	3.62	4.41	2.86	2.10
M, 15°C, 48 hours	WN	3.26	4.48	3.76	3.05
M, 15°C, 48 hours	NN	3.93	6.60	3.37	2.69
H, 15°C, 48 hours	WN	3.73	4.01	2.75	2.39
H, 15°C, 48 hours	NN	4.23	2.91	0.97	2.27
M, 28°C, 48 hours	WN	4.63	5.89	4.50	9.19
M, 28°C, 48 hours	NN	6.00	8.04	3.50	2.94
H, 28°C, 48 hours	WN	6.10	4.16	4.40	4.50
H, 28°C, 48 hours	NN	4.40	6.38	3.15	2.87

†H: hermaphrodite; M: male.
††WN: with nutrients; NN: no nutrients.

Table 5. Pollen germination percentage with saccharose concentrations of 3, 5 and 10% (date: 6 June, 1997)

Pollen source and culture conditions†	Culture medium						
	Presence of nutrients††	Saccharose (3%)		Saccharose (5%)		Saccharose (10%)	
		Agar 1.0 %	Agar 1.5 %	Agar 1.0 %	Agar 1.5 %	Agar 1.0 %	Agar 1.5 %
M, 15°C, 24 hours	WN	2.88	2.40	6.80	7.60	6.52	7.10
H, 15°C, 24 hours	WN	1.00	0.20	7.72	9.42	10.66	5.44
M, 28°C, 24 hours	WN	1.58	2.86	7.47	12.62	15.02	18.63
H, 28°C, 24 hours	WN	4.29	0.02	17.94	10.33	5.46	14.92
M, 15°C, 24 hours	NN	8.00	0.40	2.03	2.32	5.82	7.75
H, 15°C, 24 hours	NN	1.20	0.40	4.45	2.80	8.36	2.80
M, 28°C, 24 hours	NN	2.78	0.02	5.60	5.78	9.03	3.83
H, 28°C, 24 hours	NN	1.26	0.02	11.60	5.75	6.10	5.37
M, 15°C, 48 hours	WN	4.34	3.20	7.80	7.64	6.56	7.23
H, 15°C, 48 hours	WN	1.02	0.40	11.20	11.44	14.85	7.22
M, 28°C, 48 hours	WN	2.68	3.47	12.84	16.55	21.90	22.81
H, 28°C, 48 hours	WN	6.17	0.24	24.93	14.69	16.48	17.67
M, 15°C, 48 hours	NN	10.60	0.46	2.61	3.40	7.89	8.60
H, 15°C, 48 hours	NN	3.80	0.44	5.19	1.00	10.40	4.90
M, 28°C, 48 hours	NN	3.70	0.71	6.18	6.09	12.68	12.17
H, 28°C, 48 hours	NN	4.08	0.49	12.28	12.25	11.76	9.03

†H: hermaphrodite; M: male.
††WN: with nutrients; NN: no nutrients.

The second test was carried out on 5 June 1996. The germination percentage achieved was low, due to the fact that the medium used is appropriate for *in vitro* cultures for other plant tissue, and not specifically for pollen.

In the analysis of the variance of the results in Table 4, there are not significant differences between the two concentrations of agar tested (0.6% and 0.8%).

The presence of nutrients produces significant differences, with a 95% confidence level, even though the concentration used was very low (0.2 ml/100 ml of medium).

The differences are highly significant in the level of saccharose (99% confidence level), by which we interpret that levels as low as 1% and 3% are insufficient to achieve a high level of germination, and the higher of these two levels (3%) produces a greater germination percentage than the lower level (1%); this in confirmed by the third test, carried out on 6 June 1977, with saccharose levels of 3, 5 and 10%.

The time factor also gives highly significant differences (N.S.: 1%), with higher germination percentages at 48 h than at 24 h.

The temperature also has a highly significant influence (N.S.: 1%), with a greater germination percentage at 28°C than at 15°C.

In relation to the sex factor, the differences are highly significant (N.S.: 1%) when low saccharose concentrations are used, with the "male" flowers giving a higher germination percentage than the "hermaphrodites". However, with high saccharose concentrations (10%) there are not important differences between "male" and "hermaphrodite" flowers.

In the analysis of the variance of the results in Table 5, we observe with a 99% confidence level that the 5% and 10% saccharose concentrations produce significantly higher germination percentages than when the medium contains a 3% saccharose concentration. This same analysis with a 90% confidence level shows significant differences between the three saccharose levels tested (3, 5 and 10%), with the highest germination rates for 10% saccharose; this confirms the results of Tables 3 and 4, and also indicates that the optimum saccharose level for pomegranate pollen germination is around 10%.

In the third experiment, agar levels were also tested (1% and 1.5%), and this factor proved to be statistically significant (N.S.: 5%), with 1% concentration giving a better germination percentage than 1.5%, which coincides with the results of the first experiment.

The temperature factor also gives significant differences, with higher germination percentages at 28°C than at 15°C, as also shown in Test No. 2.

The time factor gives highly significant differences (N.S.: 1%), with a greater germination percentage at 48 h than at 24 h, as in the two previous experiments.

Finally, the sex variable did not show statistically significant differences, with germination percentages similar for "hermaphrodite" and "male" flowers, which contradicts the results obtained in Test No. 2 (low concentration medium), as we have already indicated.

Conclusions

(i) The incorporation of nutrients (calcium and boron) significantly increases the germination percentage in pomegranate pollen.

(ii) Agar concentrations of 1% produce higher pollen germination than 1.5%.

(iii) Saccharose concentrations should preferably be high, with no significant differences found between 10-20%; however, highly significant differences are found at low levels (1-3%). With 3, 5 and 10% saccharose concentrations, highly significant differences were found between 3 and 5%, and

almost significant differences between 5 and 10%. This shows the importance of high saccharose concentrations, with the optimum value being around 10%.

(iv) Greater germination percentages are given at 48 h than at 24 h.

(v) Higher germination percentages are obtained at 28°C than at 15°C.

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