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ABIOTIC FACTORS AFFECTING *IN VITRO* POLLEN GRAIN GERMINATION IN SAINFOIN (*ONOBRYCHIS VICIIFOLIA* SCOP.)

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Abstract

A reliable *in vitro* method for germination of sainfoin pollen grains has been developed. Highest pollen germination rate of 96% with mean pollen tube length of 223.5 μm was obtained on medium containing of 100 g/l sucrose, 200 mg/l KNO_3, 150 mg/l H_3BO_3, 150 mg/l $MgSO_4$, 150 mg/l $Ca(NO_3)_2$, 175 g/l PEG 4000 and stigma extract at pH 6.5.

Introduction

Sainfoin (*Onobrychis viciifolia* Scop.), is a perennial legume with stout erect stems from a much branched crown and deep tap roots that hold soil firmly to the deeper layers and prevents erosion. It is widely adapted to calcareous, chalky, well drained, poor and dry soils and is highly drought resistant. It has been grown in some parts of Eastern Europe and Western Asia since centuries. Sainfoin improves soil fertility by fixing atmospheric nitrogen. It can replace alfalfa on dry soils, where production of hay is limited due to shortage of water. It produces bloat safe forage and is highly nutritional. Limited improvement through conventional breeding methods has made it necessary to breed sainfoin varieties resistant against insect pests and diseases. *In vitro* study of pollen behavior helps to make viable crosses for developing appropriate breeding strategies to produce genetically diverse and high quality crops. This also assists in identifying viability, physiological and biochemical processes involved in fertilization (Pfahler *et al.*, 1997). The preservation, longevity, viability and self incompatibility of pollens differ among plant species.

Self fertility in sainfoin ranges from 0 to 37% (Bosca & Hejja 1963). Kropacova (1960) found increased efficiency of seed set from bee pollinated flowers of sainfoin. No information is available about *In vitro* pollen viability and germination of sainfoin. The present study was therefore carried out to develop a satisfactory protocol for pollen viability, germination and tube growth for its use in the breeding programs.

Materials and Methods

Plant material: Pollen grains were collected from field grown plants of an ecotype of *Onobrychis viciifolia* largely cultivated in Turkey, by gentle brushing of anthers onto Petri dishes at anthesis during morning hours in early May. Thereafter the desiccation and loss of initial pollen viability was avoided by placing a moistened filter paper inside each Petri dish before bringing them to the laboratory for analysis as described by Johri & Vasil (1961) and Shivanna & Johri (1989).

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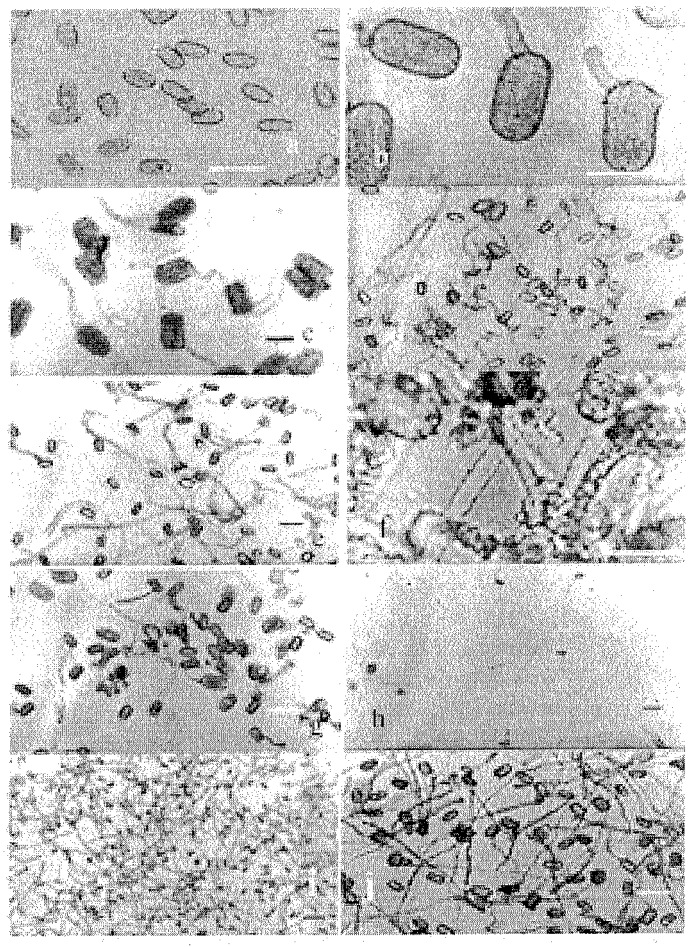

Fig. 1. *In vitro* pollen germination in Sainfoin (*Onobrychis viciifolia* Scop.) (a,b) Development of pollen tube 10 minutes after culture (c) Development of double pollen tube. Abnormalities due to addition of PEG, (d) Ejaculation of cytoplasm (from pollen), (e) Ejaculation of cytoplasm, (f) Coagulation of nutrient media and (g) Bursting of pollen grains. (h) The development of longest pollen tube in a medium containing 200 mg/l H_3BO_3, (i, j) Best pollen germination in a medium containing 100 g/l sucrose, 200 mg/l KNO_3, 150 mg/l H_3BO_3, 150 mg/l $MgSO_4$, 150 mg/l $Ca(NO_3)_2$, 175 mg/l PEG and stigma extract at pH 6.50. Bar=10 μm.

Pollen germination medium: Media reported to germinate pollen grains of *Capsicum annum* (Mercado *et al.*, 1994), *Helianthus annus* L. (Murthy *et al.*, 1994) and *Fagopyrum esculentum* (Adhikari & Campbell, 1998) had a common medium containing 100 g/l sucrose, 200 mg/l KNO_3, 150 mg/l H_3BO_3, 150 mg/l $Ca(NO_3)_2$. $4H_2O$, 200 mg/l $MgSO_4$, 300 g/l PEG 4000 and stigma extract. Therefore, sainfoin media was optimized by modifying the concentrations of sucrose (0-400 g/l), KNO_3 (0-300mg/l), H_3BO_3 (0-300mg/l), $Ca(NO_3)_2$ (0-300mg/l), $MgSO_4$ (0-300mg/l), PEG 4000 (0-600 g/l) and pH (4.00-7.50). pH was adjusted with 1N NaOH or 1N HCl. Double distilled water was used in all media preparations.

Preparation of stigma extract: On an average 150-200 stigmas were collected and squashed in mortar by addition of 3 ml germination medium and centrifuged for 5 min., at 5000 rpm, so that each of the 5 ml germination medium contained 25 µl stigma extract.

Pollen germination: The hanging drop technique was employed for the germination tests by using clear Plexiglas microscope slides with 3 mm deep built in central circular cavities. Pollen grains were shed on a drop of pollen germination medium on a cover-slip which was then turned upside down and placed over the cavity of respective slide previously encircled with commercial vaseline (Lido-Turkey) to make it air-tight. Thereafter, each of the prepared slides were placed in closed Petri dishes (100 x 10 mm) containing a water saturated paper towel to maintain moisture followed by one hour incubation at 24±1^{0}C under cool white fluorescent light (35 µmol $m^{-2}s^{-1}$). Germination was observed under light microscope (Olympus BH-2). Pollen grains with pollen tube longer than the diameter of pollen were considered as germinated (Adhikari & Campbell, 1998). Burst pollen grains were scored as ungerminated.

Statistical analysis: The data presented for pollen germination and pollen tube growth are averages obtained from 4 replications each containing 100 pollen grains. Significance was determined by analysis of variance (ANOVA) and the difference between means were compared by Duncan's Multiple Range test or T test using SPSS base 8.0 statistical software (SPSS Inc. 1998). Data presented in percentages were transformed by arcsines ($\sqrt{x}$) before statistical analysis (Snedecor & Cochran, 1967).

Result and Discussion

Pollen grains began to germinate 10 minutes after culture and germination was scored after 60 minutes (Fig. 1 a and 1b). Some pollen grains produced double tubes of approximately the same length and in others exuded mass resembled pollen tubes (Fig. 1c).

Effect of sucrose: Pollen grains of some plant species germinate and grow pollen tubes even in plain water. Some germinate in minimal media composed of sucrose and boric acid, while others need an additional complex medium with a well balanced sugar/salt mixture (Reed *et al.*, 1993, a,b). In the present study, pollen grains were subjected to 5 levels of sucrose ranging from 0 - 150 g/l in 200 g/l PEG, 150 mg/l H_3BO_3, 200 mg/l $MgSO_4$ and 150 mg/l $Ca(NO_3)_2$ at pH 6.00 with stigma extract. Sucrose levels significantly affected the germination rate and pollen tube length (Table 1; $p<0.05$). The highest germination rate (89 %) was obtained in a medium containing 100 g/l (10%) sucrose. The pollen tube grew as long as 141.0 µm. When sucrose concentrations increased to 150 g/l or decreased to 50 g/l or no sucrose used in the media, it had significantly decreased germination with corresponding reduction in length of pollen tubes (Fig. 2a). The results are similar to that of Stott (1972) who reported variation of 10-15% in sucrose levels for achieving best pollen germination among various plant species.

Table 1. Effect of various levels of sucrose on pollen germination rate and pollen tube length.

Sucrose levels (g/l)	Germination rate (%)	Pollen tube length (μm)
0	40 d[1]	115.1 b
50	58 c	129.5 ba
75	80 b	127.1 ba
100	89 a	141.0 a
150	10 e	64.0 c

Each value is the mean of 4 replicates each with 100 pollens
[1]Values within a column followed by different letters are significantly different at 0.05 level of significance using Duncan's Multiple Range Test.

Table 2. Effect of various PEG concentrations on pollen germination rate and pollen tube length.

PEG concentrations (g/l)	Germination rate (%)	Pollen tube length (μm)
0	35 e[1]	68.0 e
50	29 e	67.5 e
100	62 d	122.0 c
125	72 c	152.0 b
150	86 b	203.3 a
175	93 a	149.8 b
200	86 b	90.6 d
300	0 f	0.0 f

Each value is the mean of 4 replicates each with 100 pollens
[1]Values within a column followed by different letters are significantly different at 0.05 level of significance using Duncan's Multiple Range Test.

Table 3. Effect of pH on pollen germination rate and pollen tube length.

pH	Germination rate (%)	Pollen tube length (μm)
4.00	82 ab	79.0 f
4.50	83 ab	95.8 e
5.00	86 ab	108.5 d
5.50	77 bc	147.7 b
6.00	86 ab	137.3 bc
6.50	90 a	164.6 a
7.00	67 cd	128.6 c
7.50	53 d	86.6 ef

Each value is the mean of 4 replicates with 100 pollens
[1]Values within a column followed by different letters are significantly different at 0.05 level of significance using Duncan's Multiple Range Test.

Table 4. Effect of stigma extract on pollen germination rate and pollen tube length

	Germination rate (%)	t	Pollen tube growth (μm)	t
With stigma extract	83	4.655*	165.0	2.309*
Without stigma extract	67		140.7	

* Significantly different at 0.05 level of significance

Effect of PEG: Significant effect of PEG concentrations on pollen germination and elongation of pollen tube was quite visible (Table 2, Fig 2b, $p<0.05$). Pollen grains were subjected to media containing 8 different concentration of PEG (Table 2), with 100g/l sucrose, 200 mg/l KNO_3, 150 mg/l H_3BO_3, 200 mg/l $MgSO_4$ and 150 mg/l $Ca(NO_3)_2$ at pH 6.0 with stigma extract. The optimum germination rate (93%) was obtained in a medium supplemented with 175 mg/l PEG 4000; whereas, the longest pollen tube length (203 μm) was obtained in a medium supplemented with 150 g/l PEG. Higher concentration of PEG was inhibitory to both germination and tube length (Fig. 2b). No germination was observed in a medium containing 300g/l PEG. Murthy *et al.*, (1994) observed considerable improvement in pollen germination and tube growth by increasing PEG concentration to 296 g/l. However, they found a dramatic reduction in pollen germination and tube growth by increasing PEG (6000) concentration from 296 to 405.4 g/l. Janes (1974) reported that absorption of PEG by plant cells is inversely related to its molecular size. Several abnormalities were observed during the study which included coagulation of nutrient media, ejaculation of cytoplasm (from pollen) and bursting of pollen grains at 0, 50 and 100 g/l PEG doses (Fig. 1d,e,f and g). This indicates that up to 100 g/l PEG in the medium, osmotic pressure of medium remains lower than that of pollen. This results in swelling and imbibing of pollen with external water and bursting of pollen walls. This is contrary to the findings of Adhikari & Campbell (1998) who used PEG (6000) and suggested imbalance of osmotic pressure resulting in bursting of pollen grains due to forcing in of water from outside. Variation in results might be due to different PEG used in both experiments.

Effect of pH: Maximum of 90% pollen germination was obtained at pH 6.5 on medium containing 175g/l PEG, 200 mg/l KNO_3, 150 mg/l H_3BO_3, 200 mg/l $MgSO_4$ and 150 mg/l $Ca(NO_3)_2$ with stigma extract (Table 3; Fig. 2c). No significant changes were observed at pH lower than 6.5. Higher pH (> 6.5) resulted in decrease of germination from 90% to 53%. The longest pollen tube (164.6 μm) was observed at pH 6.5. Adhikari & Campbell (1998) reported that maximum pollen germination of buckwheat occurred at pH 5.0. It is concluded that effect of pH on germination is possibly related to the pH of the stigma before pollination. Sato *et al.*, (1998) observed fluctuating germination rate of 10-80% in *Brassica rapa* at pH 8.0.

Effect of stigma extract: The data related to germination rate and pollen tube length is shown in Table 4. Results analyzed by t test showed significant effect of stigma extract in germination media containing 200g/l PEG, 200 mg/l KNO_3, 150 mg/l H_3BO_3, 200 mg/l $MgSO_4$, 150 mg/l $Ca(NO_3)_2$, pH=6.0. The medium with stigma extract resulted in maximum of 83% pollen germination and 165 μm of pollen tube length. Medium without stigma extract had 67% pollen germination and pollen tube length of 140.7 μm. Results showed that stigma extract in the medium increases the germination rate. Germination and growth of trinucleate pollen is heterotrophic and second mitotic division in pollen tube before anthesis deprive them of several nutrients essential for germination and tube growth. This shows that specific nutrients present in stigma combined with the pollen activity and pollen tube derived hydrolytic enzymes interact positively showing synergetic effects on frequency of pollen germination and pollen tube growth. Escobar *et al.*, (1983) and Murthy *et al.*, (1994) observed that stigma extract in the medium increased pollen germination in olive and sunflower respectively. Our results are in agreement with the above findings.

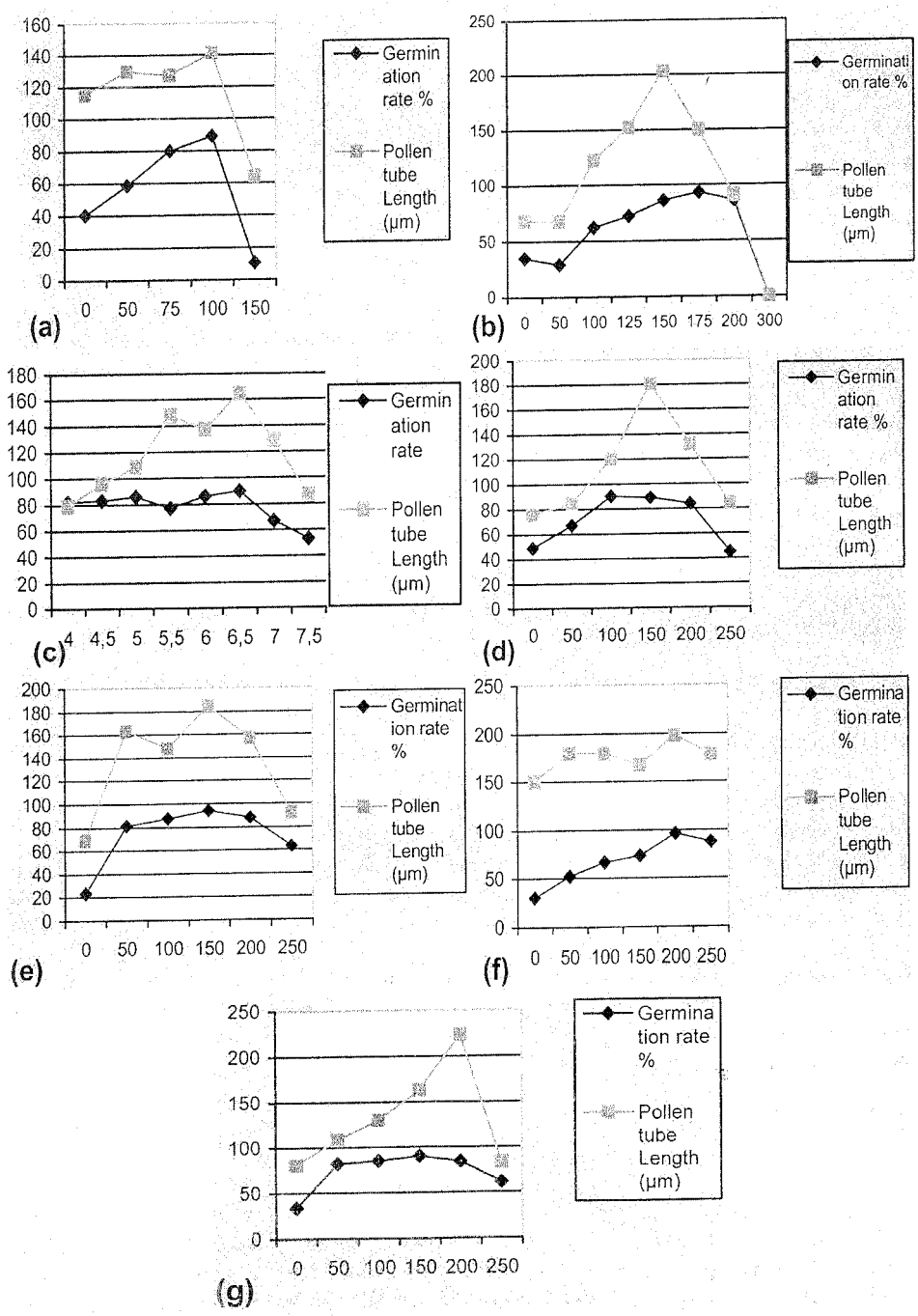

Fig. 2. Schematic representation of frequency of pollen germination and pollen tube development under various levels of (a) sucrose, (b) PEG 4000, (c) pH, (d) $Ca(NO_3)_2$, (e) $MgSO_4$, (f) KNO_3 and (g) H_3BO_3.

Table 5. Effect of salts and H_3BO_3 on pollen germination rate and pollen tube length.

	$Ca(NO_3)_2$		$MgSO_4$		KNO_3		H_3BO_3	
Concentration of salts (mg/l)	Germination rate (%)	Pollen tube length (μm)	Germination rate (%)	Pollen tube length (μm)	Germination rate (%)	Pollen tube length (μm)	Germination rate (%)	Pollen tube length (μm)
0	49.00 d	76.00 c	23.00 d	69.50 d	30.00 d	150.20 c	33.00 d	80.70 d
50	67.00 c	84.80 c	81.00 b	163.50 b	52.00 d	179.80 ab	82.00 b	108.20 cd
100	90.00 a	119.70 b	87.00 ab	148.20 b	67.00 c	179.80 ab	85.00 ab	130.00 bc
150	89.00 a	180.70 a	94.00 a	184.30 a	73.00 c	166.90 bc	90.00 a	163.20 b
200	84.00 b	131.50 b	88.00 ab	157.40 b	96.00 a	197.90 a	84.00 ab	223.50 a
250	45.00 d	84.10 c	63.50 c	91.80 c	87.00 b	177.60 ab	61.00 c	83.40 d

[1]Each value is the mean of 4 replicates with 100 pollens

[2]Values with in a column followed by different letters are significantly different at 0.05 level of significance

Effect of salts and H_3BO_3: Alterations in individual salts or H_3BO_3 levels in the media significantly affected germination (Table 5; Fig. 2d, 2e, 2f and 2g). Maximum pollen tube length was recorded in a medium containing 150 mg/l $CaNO_3$, 150 mg/l $MgSO_4$, 200 mg/l H_3BO_3 (Fig. 1h) and 100 mg/l KNO_3. The highest germination rate was achieved at 100 mg/l $Ca(NO_3)_2$, 150 mg/l $MgSO_4$, 150 mg/l H_3BO_3 and 200 mg/l KNO_3 (Fig. 1 i and j). Chang & Struckmeyer (1976), Sangduen *et al.*, (1983), Kim *et al.*, (1985); Vasilikalis & Porlings (1985) showed that any individual salt or H_3BO_3 added to germination medium significantly affected germination percentage and pollen tube development.

Various methods to assess pollen viability have been developed and used. However, there is no universal viability test which is quick, simple and reliable (Shivanna & Rangasawamy, 1992). The reported protocol is the optimization of one of the most commonly used tests. It is highly repeatable and reproducible. It can be successfully used for screening of pollen viability for use in crosses in breeding programs. The results suggest that altered concentrations of sucrose, salts, H_3BO_3, PEG, and pH with stigma extract behave variably. The differences were visible both for germination and pollen tube growth. Stanley (1974) suggested that pollen tubes grown *in vitro* stops growing before reaching the length normally required to fertilize *in vivo* and the rate of pollen tube growth is not as rapid as *in vivo*. Obviously this is mainly due to the conditions and nutrients provided by stigma that are difficult to be supplied under *in vitro* conditions.

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