AT WHAT PHENOLOGICAL PHASE IS THE STIGMA OF ARGAN (*ARGANIA SPINOSA* (L.) SKEELS) FLOWER RECEPTIVE TO POLLEN ADHESION AND GERMINATION?

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ABSTRACT

Because argan (*Argania spinosa* (L.) Skeels) reproductive biology is not yet fully understood, this study aimed to determine the flower phase when stigma is the most receptive to pollen adhesion and germination under natural pollination. An average of 9.9 from 10 stigmas were bearing pollen at the blooming flower phase (FE), whereas 6.5 stigmas were covered with pollen at the flower bud with an emerging style phase (BFS). Although the number of deposited or adhered pollen grains did not differ between both phenological phases, only an average of 1.6 pollen grains would germinate on BFS stigmas as compared to 6.4 on FE. More over, highly significant differences were observed between phenological phases for stigma height, pollen tube number per stigma, maximum pollen tube length and maximum pollen tube length to stigma height ratio, FE being the phase showing the highest values for these variables. No pollen germination was observed on the flower bud (BF) stigmas, and pollen tubes, although present on BFS, never attained the base of its style reaching the ovule. However pollen tubes reached the base of the style in 25 % of FE. Therefor, FE seems to be the phase, when, most likely, stigma is to be receptive to pollen. To avoid any possible contamination during artificial pollination, however, emasculation should be carried out at early BF phase as a precaution, because pollen may germinate on BFS stigma as well. Tree and phenological phase × tree interaction were significant for all variables. Therefore, the variability of pollen adhering and germination among individual trees may indicate a limitation of allele exchange among genotypes because of differential stigma receptivity and male gametophytes viability adding to the previously encountered time diversity of FE number and pollen fertility.

Key words: *Argania spinosa*, flower, phenological phases, pollen tube, pollination, variability

INTRODUCTION

Argan tree (*Argania spinosa* (L.) Skeels) is adapted to aridity in the south west of Morocco (EMBERGER 1939; PRENDERGAST & WALKER 1992; LE HOUÉROU 1989; FERRADOUS *et al.* 1996; ZAHIDI & BANI-AAMEUR 1999a and b). It flowers and fruits with as little as 100 mm rainfall (PERROT 1907; METRO 1952; FERRADOUS *et al.* 1996; BANI-AAMEUR *et al.* 1998; BENLAHBIL & BANI-AAMEUR 1999; BANI-AAMEUR 2000; BANI-AAMEUR 2002a). However large variability of flowering intensity was observed among climatic years, sites, tree genotypes and shoot types. In any case, the peak of flowering occurs in spring

Argan inflorescence is a glomerule of up to 15 pentamerous hermaphroditic flowers. They are grouped in the axils of the leaves or on the nodes of the shoots. The glomerule may include all phenological phases, i.e. flower bud – BF (1 to 2 mm), flower bud with an emerging style – BFS (1 to 2 mm), blooming flower FE (2 to 3.15 mm), dry flower with corolla – FSCP (2 to

3.15 mm) and finally, dry flower without corolla – FSC (1 to 2 mm), as well as young fruit. Anthesis, which may occur at BF, is complete at BFS phase, while the anthers are still covered with the perianth (BELMOUDEN & BANI-AAMEUR 1995, DEROIN & BANI-AAMEUR 1999). Pollen fertility, using acetocarmine staining, varied from 100 to 49.1 % (BANI-AAMEUR 2002b). Its size varied from 17 to 32 µm and germination pore number varied from two to six. Tested *in vitro*, pollen is not completely ripe for germination before FE phase (BENLAHBIL & BANI-AAMEUR 2002).

Pollination includes male/female interactive processes where pollen germination on the stigma is a succession of stages from pollen adhesion to reaching the ovule by the pollen tube for fecundation (ULRICH 1952). However, argan pollination behaviour is not understood yet. Especially, flower stigma receptivity is not clearly established, what is a limitation for setting up a method of artificial pollination to facilitate the crossing of selected genotypes. Our overall goal in this study is to determine the flower phase when stigma is

the most receptive to pollen adhesion and germination. In the course of three kinds of observation, we aimed to establish at which flower phenological phase the stigma receptivity is the most favourable to pollination.

MATERIALS AND METHODS

Plant material

The site of investigations was located at Ait Melloul, South west of Morocco. Sampling took place at the peak of flowering in spring 1997 for the first two kinds of observation and in spring 2001 for the last one. Flowers were collected at random when the phenological phases were at their full development, i.e. BF was collected just before reaching BFS, this phase was observed just before blooming and FE was collected at full bloom.

In situ observation of pollen deposition

To examine the degree of exposure of phenological phases to pollen reception, we examined in the field the presence or the absence of pollen on the stigma. Four replications of each of 10 BFS and 10 FE flowers of three random trees were observed using a field magnifier (G × 20).

Light microscope observation of pollen adhesion

Pistils of ten BFS flowers and ten FE flowers from three random trees were excised using a laboratory magnifier (G × 20). The stigmas were agitated in a drop of acetocarmine on a slide to separate pollen covering the stigma but not adhering to it. The pistils were cut longitudinally with a razor blade and the stylar canal was stained with acetocarmine under a light microscope (G × 40).Then it was possible to count both adhered and germinating pollen grains (ASCHER & PELOQUIN 1968; RAMSEY &VAUGHTON 2000).

Fluorescent microscope observation of pollen tube growth

To determine in vivo pollen growth, pistils from ten BF, ten BFS flowers and ten FE flowers collected from six random trees were *in situ* excised using a field head magnifier (G × 20). They were immediately fixed with a mixture of formaldehyde, ethanol 80 % and glacial acetic acid (ratio 1: 8: 1) and stored for 24 hours at 4 °C (MARTIN 1958; TANGMITCHAROEN & OWENS 1997; KALINGANIRE *et al.* 2000). Thereafter the pistils were rinsed with distilled water for five minutes and cleared

in 8 M NaOH for 96 hours at room temperature until most of the tissues became transparent. They were then rinsed again in distilled water for five minutes, mounted on a slide and stained with 0.1 % aniline blue in 0.1 M K_3PO_4. They were observed using an Olympus BH2-RFC photomicroscope with UVFL objectives and barrier filter L-435, fluorescence with 405 nm excitation and 490 nm barrier filters at G × 100. Style height, maximum pollen tube length and pollen tube number per stigma were measured.

Data analysis

ANOVA was a two-factors design of trees and phenological phases (SOKAL & ROHLF 1995) In the first kind of observation, it was performed on the number of stigmas covered with pollen for four replications of ten flowers. In the second case, it concerned the number of pollen grains on the stigmas, adhering to their surface or germinating on it using ten flowers as replications. Finally, in the third kind of investigation, it was performed on stigma height, maximum pollen tube length, pollen tube number per stigma as well as on maximum pollen tube length to stigma height ratio using ten flowers as replications. In this case, maximum pollen tube length to stigma height ratio was subject to a $\sqrt{(x + 0.5)}$ transformation. The Least Significant Difference test (LSD, $\alpha = 5$ %) of equality of means was used to compare differences between means. Statistix (Analytical Software) software was used for computation.

RESULTS

In situ observation of pollen deposition

Phenological phase was a highly significant main factor for the number of stigmas bearing pollen, whereas neither tree nor interaction was significant (Table 1). Mean number of stigmas bearing pollen was 9.9 for FE

Table 1. Analysis of variance of the number of stigmas with the deposit pollen for three argan trees and two phenological phases under natural conditions.

Source of variation	DF	Mean square
Tree	2	1.17 ns
Phase	1	70.04 **
Tree × phase	2	2.17 ns
Error	18	0.95

DF: degrees of freedom; **: significant at 0.01 level; ns: non significant

contrasting with 6.5 stigmas for BFS. Note that argan stigma is dry.

Light microscope observation of pollen adhesion

Phenological phase was a highly significant main factor for the number of pollen grains germinating on the stigmas, whereas the factor tree was significant for the number of deposited pollen grains (Figure 1A, Table 2). Interaction was not significant for anyone of the observed variables. Both BFS and FE received similar amounts of deposited pollen, although these numbers varied between 16 and 70 grains depending on the tree (Table 3). But an average of 6.4 grains would germinate on FE stigmas as compared to 1.6 for BFS.

Fluorescent microscope observation of pollen tube growth

Phenological phase was a highly significant main factor

for stigma height, pollen tube number per stigma, maximum pollen tube length as well as the maximum pollen tube length to stigma height ratio (Table 4). Tree was significant for maximum pollen tube length and for maximum pollen tube length to stigma height ratio. The interaction phenological phase × tree was highly significant for pollen tube number per stigma and maximum pollen tube length. Mean style height was 3875.5 μm for FE and 3470 μm for BFS where it significantly varied among trees from 3250 μm to 3625 μm (Table 5). On average, 10 pollen grains formed pollen tubes on FE stigmas, varying from 2.4 to 21.3 among trees (Table 5). It contrasts with 1.5 pollen tubes developed in BFS, where trees did not differ significantly. Maximum pollen tube length involved on average, 68 % of FE style length (between 55 % and 85 % depending on the trees), what contrasted with 6 % in BFS. Pollen tubes never exceeded the maximum of 42.9 % of BFS style length and only 5 % of cases exceeded 25 % (thus never attaining the ovule) whereas 42 % of the exam-

Table 2. Analysis of variance of the number of pollen grains deposited on the stigma, adhered to it and germinating for three argan trees and two flower phenological phases under natural conditions.

Source of variation	DF	Mean square of the number of pollen grains		
		Deposited	Adhered	Germinating
Tree	2	18933 *	11139 ns	27.6 ns
Phase	1	13440 ns	2996.3 ns	345.6 **
Tree × phase	2	9830.2 ns	19456 ns	0.9 ns
Error	54	5131	9159	43.8

DF: degrees of freedom; ** : significant at 0.01 level; * : significant at 0.05 level; ns: non significant

Table 3. Mean, maximum and minimum deposited, adhered and germinated pollen grain number for three argan trees at two flower phenological phases BFS and FE as described in the text.

Number of pollen grains	Phase	Tree			Mean	Maximum	Minimum
		1	2	3			
Deposited	BFS	9.8	27.4	71.2	36.1	263	0
	FE	21.4	107.9	68.9	66.1	302	1
	Mean	15.6 b	67.5 a	70.05 a	51.1		
Adhered	BFS	7.2	26.1	110	47.7	56	0
	FE	36.7	39.8	24.4	33.6	88	5
	Mean	21.9	32.9	67.2	40.7		
Germinating	BFS	0	2.3	2.5	1.6 b	11	0
	FE	5.3	6.8	7.1	6.4 a	48	0
	Mean	2.7	4.6	4.8	4		

Different letters note significant differences (LSD at 0.05 level) as comparisons are made among tree means or between phenological phase means.

Table 4. Analysis of variance of pollen tube number, style height, maximum pollen tube length and maximum pollen tube length to stigma height ratio for six trees and two phenological phases under natural conditions.

Source of variation	DF	Mean square			
		Pollen tube number	Style height	Maximum pollen tube length	Maximum pollen tube length/stigma height ratio
Tree	515	11.4 ns	176.3 ns	38.2 *	8.6 *
Phase		114.9 **	6900.8 **	4555.2 **	1182.2 **
Tree × phase		7.1 **	140.8 **	11.1 ns	3.1 ns
Error	108	0.7	25	11.6	3.17

DF: degrees of freedom; ** : significant at 0,01 level; * : significant at 0,05 level; ns : non significant

Table 5. Mean, maximum and minimum pollen tube number, style height, maximum pollen tube length and maximum pollen tube length to stigma height ratio of six argan trees and two flower phenological phases, BFS and FE as described in the text.

Tree	Pollen tube number			Style height (µm)			Maximum pollen tube length (µm)			Maximum pollen tube length/stigma height ratio		
	BFS	FE	Mean	BFS	FE	Mean	BFS	FE	Mean	BFS	FE	Mean
1	0.6	7.5 b	4.1	3250.0 d	3837.5	3545.0	21.9	2385.0 bc	1245.0 b	3.0	62.0 bc	32.5 b
2	0.6	2.4 c	1.5	3437.5 bc	3825.0	3632.5	27.3	2110.0 c	1125.0 b	4.0	54.7 c	29.4 b
3	1.7	18.5 a	10.1	3400.0 c	3875.0	3637.5	44.3	3215.0 ab	1732.0 a	6.6	82.8 ab	44.7 a
4	1	2.6 c	1.8	3550.0 ab	3787.5	3670.0	47.2	2135.0 c	1217.0 b	8.1	56.0 c	32.1 a
5	2.9	8.3 b	5.6	3550.0 ab	3862.5	3707.5	50.3	2655.0 bc	1475.0 ab	8.1	68.5abc	38.3 ab
6	2.3	21.3 a	11.8	3625.0 a	3900.0	3762.5	49.6	3335.0 a	1849.0 a	8.5	85.3 a	46.9 a
Mean	1.5 b	10.1 a	5.8	3470.0 b	3847.5 a	3659.0	40.1 b	2639.0 a	1440.4	6.4 b	68.2 a	37.3
Max	10	31		3750.0	4000.0		1450.0	4000		42.9	100.0	
Min	0	1		3000.0	3625.0		0.0	25.0		0.0	6.5	

Different letters note significant differences (LSD at 0.05 level) as comparisons are made among tree means.

ined BFS did not show pollen tubes (Figure +B, C, D, E, F). In contrast, every examined FE showed at least one pollen tube and ten tubes were observed in 50 % of the cases, and the pollen tubes attained the ovule in 15 % of FE.

DISCUSSION AND CONCLUSION

When the stigma has been exposed to pollen, both BFS and FE may present similar numbers of deposited and adhered pollen grains. Contrasting with up to 100 % of FE, however, only 40 % of the exposed BFS stigma received pollen on average. In addition, whereas many pollen grains would germinate on FE stigmas, no pollen at all was observed at the germination stage on those of

BF. Argan is an outcrossing species (BENLAHBIL & BANI-AAMEUR 1999), and has a dry stigma. Although the outcrossing species would display the stronger adhesion to enforce their competence to attach pollen, the dry stigmas, as it has been observed for *Arabidopsis* Heyneh., are usually barriers to the mass pollination (ZINKL & PREUSS 2000). They promote pollen adhesion, pollen hydration and pollen tube penetration, but the rapid pollen hydration requires the direct contact between the stigma cells and the pollen grain, facilitating water transfer through instant fissures in the stigma cell wall. To make the pollen-stigma interaction possible, these processes require activation of specific stigma genes (ZINKL *et al.* 1999). The BF phase is probably not ready for such an activation, which commences at the next stage (BFS) to be fully achieved
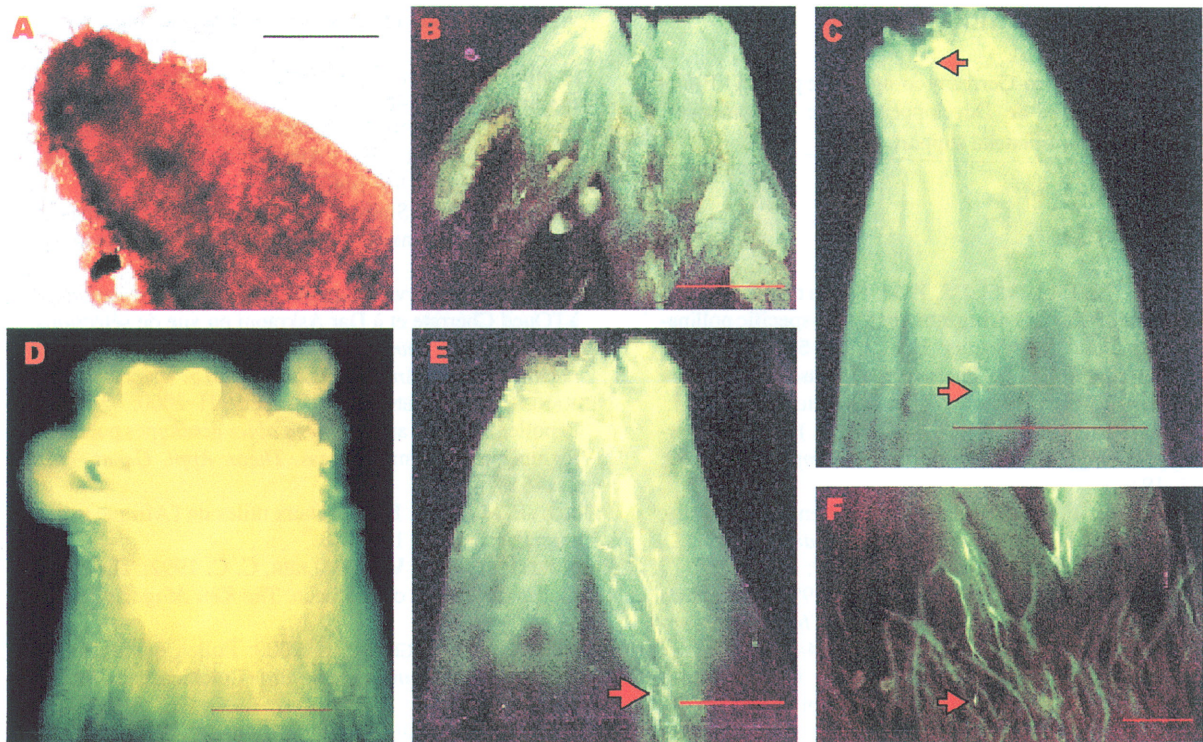
Figure 1. Stigmas of the flower of *Argania spinosa* (scale = 50 µm) stained with acetocarmine showing adhered pollen grains at FE phase (A); stained with wit fluorescent aniline blue at BF phase when no pollen adhered or germinated (B), at BFS phase when few pollen grains germinated but never reached the base of the style as traced by the arrows (C), and at FE phase when large number of pollen grains germinated elongating (D, E), and many of them attained the ovule at the base of the style (F).

in FE phase. However further research is needed to support these inferences and to determine fine argan pollination mechanisms under arid conditions.

BF phase was the most abundant and BFS and FE were the least abundant (BANI-AAMEUR 2000). In addition, BFS was observed in some trees only. For these individuals, there is a hesitation in determining the origin of the pollen that germinated on the BFS stigmas. From the previous studies we know that anthesis occurs at BF and is complete at BFS, shedding pollen from anthers inside the closed flower bud (BELMOUDEN & BANI-AAMEUR 1995; DEROIN & BANI-AAMEUR 1999). However, argan pollination is mostly outcrossing (BENLAHBIL & BANI-AAMEUR 1999). The pollen from BF does not germinate at all (BENLAHBIL & BANI-AAMEUR 2002). Although the pollen from FE was proved to be the most fertile, a low percentage of BFS pollen germinates as well. Therefore, it seems to be doubtless that the pollen encountered on BFS stigmas comes from the self-pollination, while the stigma is still confined within BF. Contrariwise, the pollen observed on the exposed stigma of BFS or FE, originates probably from the cross-pollination by insects or wind.

This study showed that BFS stigma may be receptive and pollen may adhere to it and germinate. Thus, to avoid any possible contamination during artificial pollination, emasculation should be carried out at early BF phase before pollen shedding.

Compiling information from previous studies and from this study, it is now determined that the FE stage was at its population maximum for only three weeks during spring (BANI-AAMEUR 2000; BANI-AAMEUR 2002a, BENLAHBIL & BANI-AAMEUR 2002). This period corresponds to the availability of the fertile pollen and the receptive stigmas. For these three weeks during spring, the conditions are favourable to maximum within–population gene flow. However, the large variability observed among tree genotypes for glomerule count (BANI-AAMEUR 2002a), FE number at a particular sampling date (BANI-AAMEUR 2000) and pollen adhering and germination (present study), suggests that there may be a limitation of allele exchange among genotypes because of different timing of stigma receptivity and male gametophyte viability as it was previously observed for other species (NIKKANEN *et al.* 2000).

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