South African Journal of Botany 137 (2021) 440–450

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

Reproductive biology, phenology, pollen viability and germinability in Kersting's groundnut (*Macrotyloma geocarpum* (Harms) Maréchal & Baudet, Fabaceae)

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ARTICLE INFO

Article History:
Received 30 September 2020
Revised 10 November 2020
Accepted 17 November 2020
Available online xxx

Edited by S-L Steenhuisen

Keywords:
Style movement
Protogyny
Forced self-pollination
Anther dehiscence
Stigma receptivity
Pollen tube growth

ABSTRACT

Knowledge gap on the reproductive biology of orphan crops is a major challenge to their cultivar development and genetic improvement. This study described the reproductive organs and phenology, assessed receptivity of stigma versus anther dehiscence, and examined pollen viability and germination in Kersting's groundnut. Experiments were conducted on nine morphotypes in a randomized complete block design with three replications in a screenhouse. Phenological observations were made on 1026 flower buds. Anther dehiscence was determined through microscopic observations while receptivity of the stigma was assessed using the hydrogen peroxide test. Pollen viability was assayed using histochemical staining. *In vitro* germination and pollen tube growth were assessed for up to 72h. Flowers were bisexual and incompletely protogynous, and spontaneous self-pollination was favoured by style bending. Flowering and fruiting were classified into six developmental stages each. Timing of all stages differed significantly ($P<0.001$) among morphotypes. The stigma was receptive 1-2 days before anther dehiscence and remained so until each flower wilted. Anthesis started 3 days from young bud appearance and lasted between 2–4 days depending on the morphotype. Pollen viability rates were very high (88–98%) and differed among morphotypes ($P<0.001$). Pollen germination rates were low (8–37%) and varied among morphotypes and anthesis stages ($P<0.05$). Pollen tube growth varied significantly among morphotypes, anthesis stages and incubation time. The morphotype ZHLA-2 exhibited the highest reproductive vigour and could be recommended as pollen donor for hybridization. The study provides information on the best time and stage for emasculation and is expected to help breeders optimise a hybridisation protocol.

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1. Introduction

The major breakthroughs in plant genetic improvement that favoured the advent of the 'Green revolution' in the 1960s were associated with the knowledge of reproductive biology of crop species (Whitford et al. 2013; Jaiswal et al. 2016; Singh et al. 2010). Yet, the knowledge base regarding the reproductive biology of the so-called 'orphan crops' remains cursory and indubitably a hurdle for their improvement (Cullis et al. 2019). Studies related to the anthesis, pollen viability, in vitro pollen germination, and stigma receptivity have been conducted in many minor legume crops including faba bean (*Vicia faba* L.) (Stoddard 1986), cowpea (*Vigna unguiculata* L.Walp) (Ribeiro et al. 2013; Ige et al. 2011), pigeon pea (*Cajanus cajan* (L.) Millsp.) (Jayaprakash and Sabesan 2013; Kar and Datta 2017), and Bambara groundnut (*Vigna subterranean* (L.) Verdc.) (Onwubiko et al. 2011; Chandra et al. 2019; Linneman 1993) among others, and allowed important achievements in their genetic improvement. For instance, various pigeon pea hybrid varieties with 25 – 69% yield superiority over the local cultivars were obtained in

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https://doi.org/10.1016/j.sajb.2020.11.015
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India by the means of intra and interspecific crosses (Saxena 2015). In contrast, low success rates of artificial hybridization were recorded in chickpea (*Cicer arietinum* L.), and the lack of details on the flowering stages chosen for making crosses was the principal underlying cause (Kalve and Tadege 2017; Altaf and Ahmad 1990).

Kersting's groundnut (*Macrotyloma geocarpum* (Harms) Maréchal & Baudet, Fabaceae), commonly known as "doyi" in Benin, is an indigenous legume crop widely grown and consumed in West Africa. Its seeds are rich in protein, and contain essential micronutrients including key minerals (Fe, Ca, Mg, K, Zn, Na, P) and vitamins (A, B1, B2) (Chikwendu 2015). The crop is thus a valuable surrogate protein source for people with limited access to animal protein and a potential source of income for rural communities. Listed as one of the 101 promising orphan crops promoted by the African Orphan Crops Consortium (AOCC), the crop has recently received greater attention from researchers in Benin (Assogba et al. 2015; Akohoue et al. 2019; Akohoue et al. 2018; Loko et al. 2019; Coulibaly et al. 2020; Agoyi et al. 2019), Ghana (Mohammed et al. 2018; Mohammed et al. 2019; Jaiswal et al. 2019) and Nigeria (Chikwendu 2015). Studies of reproductive biology African orphan legume crops such as Kersting's groundnut are, however, still very limited.

To our knowledge, the only reproductive biology study in Kersting's groundnut was carried out two decades earlier and was based on three cultivated varieties (Obasi and Ezedinma 1994). The study showed that the time from flower initiation to anthesis was about 6 days and did not vary among varieties. Moreover, it was found that the stigma became receptive 12 hours before anthesis while anthers dehisced 7-8 hours before anthesis (Obasi and Ezedinma 1994). Unfortunately, the study did not provide any detail regarding the characteristics of the varieties studied. Yet, flower and seed morphotypes are known to impact important reproductive traits such as pollen production, pollen viability, pollen germination capacity, stigma receptivity, fruit set, and eventually grain yield as reported in pigeon pea (Kar and Datta 2017). Knowledge of these and additional reproductive characteristics in different Kersting's groundnut morphotypes will help breeders determine the optimal time and the right stage of flower bud to use to perform emasculation and pollination. This information is imperative to successful hybridization in the crop as the stage of flower bud used for emasculation and the pollination time can affect the success rate of artificial hybridization as reported in *Capsicum annuum* L. (Kivadasannavar et al. 2013) and *Arachis hypogaea* L. (Chu et al. 2016), respectively. On the other hand, the genetic dissection of complex heritable traits relies on the development of mapping populations which can only be established through crosses. Therefore, a better knowledge of the floral morphology and reproductive biology of Kersting's groundnut is a prerequisite in formulating effective strategies for improvement of the crop and understanding the genetics of complex traits such as yield components.

Therefore, the present study aimed to (i) describe the reproductive organs and phenology; (ii) assess receptivity of stigma and anther dehiscence; and (iii) examine pollen viability and germination in Kersting's groundnut.

2. Material and methods

2.1. Plant materials and experimental design

Nine accessions (Table 1) representing different morphotypes of Kersting's groundnut based on the combination of seed coat and flower colours were investigated in this study. The experiment was conducted in the screenhouse of the Laboratory of Applied Ecology (06°24′55.42″N 02°20′21.44″E; altitude 20 m.a.s.l) at the Faculty of Agronomic Sciences (University of Abomey-Calavi, Benin). Seeds were sown in plastic pots (20 cm diameter × 21 cm depth) containing sterilized soil at a rate of two seeds per pot, and seedlings were thinned to one per pot 10 days after sowing. The pots were laid out in a randomized complete block design with three replications in which each plot was made up of 5 pots aligned one next to the other. The plants were watered every 2-3 days.

2.2. Flowering and fructification phenology

Phenological observations were made to determine the timing and duration of the different flower developmental stages. In total, 1026 flower buds (38 flower buds × 9 morphotypes × 3 replications) were randomly selected and monitored daily, based on the observable changes in colour and size of floral parts. The selected flower buds were tagged at their initial stage. After wilting, tagged flowers were monitored every 2 days until pod maturation. To determine the initiation of anthesis and anther dehiscence, 432 flowers buds were tagged and monitored for eight days. Six flowers per morphotype were removed every day, then dissected and anthers observed with an Olympus stereoscopic microscope. Likewise, stigma receptivity was measured on 432 random flowers (n=48 flowers per morphotype), over a period of eight days, using the method of Dafni et al. (2005). Briefly, 1-2 drops of a hydrogen peroxide solution (H_2O_2; 3%) were put on the stigma of six flowers per morphotype per day to test a peroxidase activity indicating stigma receptivity. Stigmas that produced bubbles within 2-3 mins were considered receptive (Thimmaiah et al. 2018). Anthesis time was defined as when the stigma was receptive to pollen and anthers were ready to release it (Rudall et al. 2007).

2.3. Pollen viability

Pollen viability was tested using the Carbol Fuchsin staining method (Stanley and Linskens 1974). Nine flowers were randomly collected per morphotype at different periods (pre-anthesis, anthesis, post-anthesis), for 27 flowers sampled per morphotype in total. Then, pollen from 3 anthers picked on each flower were dusted on 3 glass slides per flower (729 slides in total) and stained with 1% Carbol Fuchsin. All slides were observed and imaged with a Carl Zeiss Primo Star Binocular Microscope (Germany). Pollen grains appearing deep red were considered as viable whereas colourless or light stained grains were considered non-viable (Gaaliche et al. 2013).

2.4. In vitro *pollen germination and pollen tube growth*

In vitro germination and pollen tube growth were assessed using the hanging drop method (Stanley and Linskens 1974). Three flowers were randomly collected per morphotype and for each of the following phases: pre-anthesis, anthesis, and post-anthesis. A small drop of germinating medium was placed onto cover glasses inside a petroleum jelly circle (Stanley and Linskens 1974). Pollen grains were sown on the drop by squashing a single anther using sterilized needles. This operation was performed under an Olympus stereoscopic microscope at 10x. Slides were delicately placed on the cover glasses, then the slides were inverted and placed into petri dishes containing moist filter paper. The germinating medium contained 5% sucrose ($C_{12}H_{22}O_{11}$), 5 ppm boric acid (H_3BO_3) and 1% agar (Gaaliche et al. 2013). Petri dishes were incubated in darkness at 30°C for 24, 48 and 72 hours. All slides were viewed and microphotographs were taken with a Carl Zeiss Primo Star Binocular Microscope. Germination percentages were determined by counting germinated and total number of pollen grains per slide. Pollen was considered germinated when its tube length exceeded the grain diameter (Salem et al. 2007). Pollen tube length was measured on 15 pollen tubes for each of the 81 treatments (3 phases × 9 morphotypes × 3 incubation periods). For this purpose, microphotographs were first assembled in mosaic using Autostitch for Macintosh (Brown and Lowe 2007; Ma et al. 2007) and pollen tube lengths were

Table 1
Kersting's groundnut morphotypes and their characteristics.

N°	Accession	Seed coat colour	Flower colour	Country of origin	Donor
1	ZHLA-2	Cream	Purple	Benin	Collected
2	GTA-3	Cream	White	Benin	Collected
3	ZHLA-1	Brown	White	Benin	Collected
4	GBO-4	Brown	Pink	Benin	Collected
5	T-10	Black	Pink	Ghana	SARI
6	BUR7	Black	White	Burkina Faso	INERA
7	E-5	Cream with black butterfly-like eye	White	Ghana	SARI
8	BUR13	Cream with black butterfly-like eye	Purple	Burkina Faso	INERA
9	TK9-12	Gray mottles on brown background	Purple	Nigeria	IITA

SARI – Savanna Agricultural Research Institute, INERA – Institut de l'Environnement et de Recherches Agricoles, IITA – International Institute of Tropical Agriculture.

subsequently measured using Fiji ImageJ version 2.0.0 (Abràmoff et al. 2004). Mosaicked images were calibrated in ImageJ before measurements.

2.5. Data analysis

Data were analysed in R version 3.6.1 (R Core Team 2020). The different flowering and fruiting phenological stages were determined and described by means of descriptive statistics (mean ± standard error, coefficient of variation, and range). Differences in the duration of each phenological stage among morphotypes were assessed using generalized linear mixed models (GLMM) with Poisson or negative binomial error distribution (in case of overdispersion) performed in the 'lme4' package (Bates et al. 2015). Overdispersion was checked using the function provided by Bolker et al. (2009) (http://bbolker.github.io/mixedmodels-misc/glmmFAQ.html#testing-for-overdispersioncomputing-overdispersion-factor). In all models, morphotype was included as a fixed effect and flowers nested within plants within blocks (*1 | Block/Plant/Flower*) were used as random effects to control the spatial repeated measures of flowers per plant across blocks, as recommended by Crawley (2012). The Tukey post-hoc test was performed to test for significant differences among morphotypes, using the 'multcomp' package (Hothorn et al. 2008). The *Anova* function from the 'car' package (Fox and Weisberg 2019) was used to extract the p-value of fitted GLMMs. Estimated marginal means and associated standard errors were extracted from the models using the 'emmeans' package (Lenth 2019) and used to describe the flowering and fruiting phenological stages, along with coefficient of variation and range. To determine the period of anthesis, as well as the onset of anther dehiscence and stigma receptivity for each morphotype, GLMMs were constructed and marginal means were estimated from the models using the 'emmeans' package (Lenth 2019). The day at which 50% of the anthers and stigmas tested positive for dehiscence and peroxidase activity, respectively, was determined and used as the basis for comparison of the morphotypes. Percentages of viable pollen were compared among anthesis stages (pre-anthesis, anthesis, post-anthesis) and morphotypes using a Generalized linear model (GLM) with quasipoisson family, performed in 'lme4' package (Bates et al. 2015). Tukey post-hoc test as performed in the 'multcomp' package (Hothorn et al. 2008) was used for multiple comparisons. Pollen germination percentage and pollen tube length were summarized using descriptive statistics (mean ± standard error, coefficient of variation and range). Differences in pollen germination rates among morphotypes, incubation time, anthesis stages and their interactions were assessed using GLM. Similarly, Analysis of variance (ANOVA) or Kruskal-Wallis test were used, when appropriate, to test

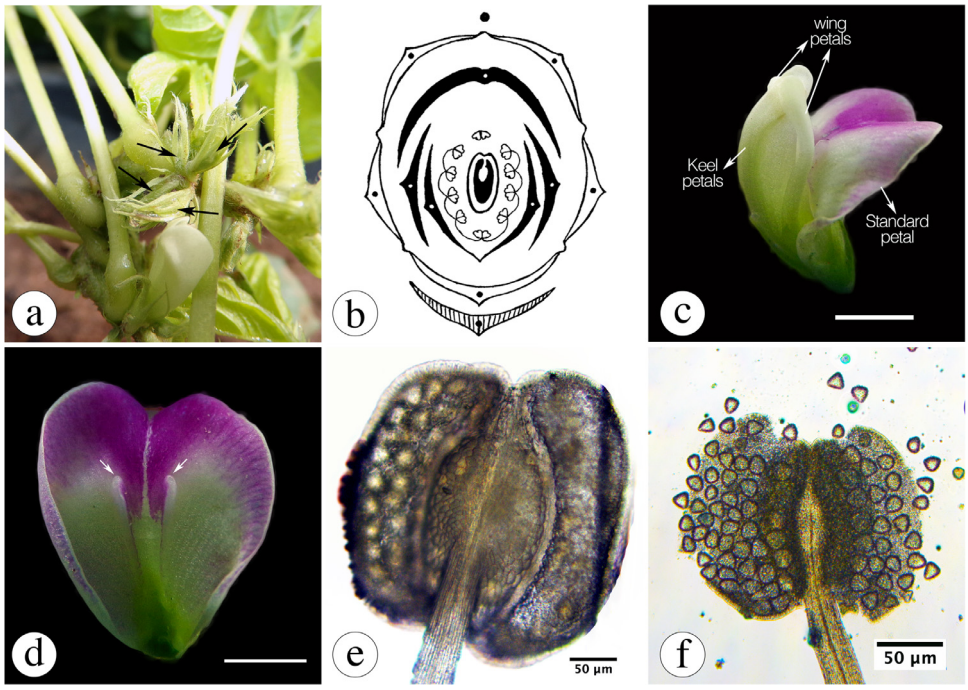

Fig. 1. Flower structure of Kersting's groundnut (*M. geocarpum*). **(a)** Emergence of four flower buds at a node, **(b)** Floral diagram, **(c)** Opened flower showing the different parts of the corolla, **(d)** Exudate glands on the inner face of the standard petal (white arrows), **(e)** Dorsifixed immature anther, **(f)** Dehiscing anther. Scale bar = 4 mm in (c) and (d).

for differences of pollen tube length among morphotypes, incubation time, and anthesis stages. ANOVA followed by Tukey post-hoc test were used when normality and homoscedasticity assumptions were met. Kruskal-Wallis test and the Dunn post-hoc test were performed when the two assumptions were violated.

3. Results

3.1. Floral morphology

The inflorescence was an axillary-pseudoraceme with four sessile or subsessile flowers at each node (Fig. 1a). Fig. 1b depicts the floral diagram of Kersting's groundnut. The floral formula is

$$\boldsymbol{BBt_2}\%\downarrow \mathbf{K}_{(5)}\boldsymbol{C}_{1+2+(2)}\boldsymbol{A}_{1+(9)\_}\boldsymbol{G} \quad (1)$$

indicating that the flower was zygomorphic (%) with one bract (B) and two persistent bracteoles (Bt). Bract and bracteoles 6-7 × 1-1.2 mm, lanceolate to linear, acuminate. The flower had a single plane of symmetry in meridian direction (↓). It was hermaphrodite (⚥) with pentamerous calyx and corolla. The calyx was green and composed of five valvate sepals (K), lanceolate, acuminate, fused towards the base, forming a calyx tube. The two vestibular lobes 5-16 × 1-9.5 mm were almost fully fused. The bracteoles were longer than the calyx tube. The corolla, pale white-green, greenish, yellowish, white tinged with purple or pink depending of the morphotype, was made of five petals (C) with vexillary arrangement: a standard petal (vexillum), two wing petals (alae) and two keel petals fused at their tips, forming a boat-shaped structure (carina) that enclosed the reproductive organs (Fig. 1c). The standard petal 9.5-19 × 6-17 mm, ovate to obovate, glabrous, had two glands that exuded a sticky substance during the early flowering stages (Fig. 1d). The wings 10-12 × 2-2.4 mm, glabrous. The keels 10-13 × 2.50-3.5 mm, glabrous and clawed. Sepals and petals alternated in position (Fig. 1b). The androecium was gamostemone, diadelphous, consisted of ten stamens 9–17 mm long, the vexillary one being free forming a falcate staminal tube. The anthers were 27.85-401.95 × 19.04-339.24 μm, ellipsoid, bilobed and dorsifixed with longitudinal dehiscence (Fig. 1e,f). Dehiscing anthers released pollen grains of 7.9-38.9 μm in diameter. The gynoecium (G) was sessile or subsessile, 8–17 mm long with an elongated superior ovary 1.2–5 mm long, containing 2 to 3 green, shiny and globular ovules with erect dorsal placentation. The stigma was capitate. Ovules diameter averaged 0.25 mm.

3.2. Phenology

3.2.1. Flowering phenology

Flowering phenology was determined based upon observable changes in colour and size of flowers. Six developmental stages including initiated flower (S1), young bud (S2), developed bud (S3), mature bud (S4), opened flower (S5) and wilted flower (S6) were identified (Fig. 2). Flower initiation (S1) was observed in average 53 days after sowing (DAS) and this stage typically lasted 6–8 days depending on the morphotype. At S1, all the flower parts were already in place (Fig. 2d), though the perianth was not developed (Fig. 2a). During this phase, the *initiated flower* elongated and gradually became a *young bud* (S2). The S2 stage occurred in average 59 DAS. At this stage, five sepals became visible. A tiny light green bud appeared between the sepals. The colour of the young bud was the same irrespective of the morphotype. The sepals protruded the bud by 3–5 mm (Fig. 2b). The stamen had a short filament carrying at the top a yellowish and already developed anther. The style was bent downwards with the stigma also curling downwards and inwards. At this phase, the stigma and part of the style are above the level of the anthers (Fig. 2e). The transition from young to *developed bud* (S3) was very fast, varying from a few hours to two days. The S3 stage was characterized by the elongation of the corolla which exceeded the calyx (Fig. 2c). The standard petal remained closed making the inner petals (wings and keels) not visible. The upper half portion of the standard started to change colour. The elongation of the stamens and pistil was noticeable, though the physical configuration of the two organs remained unchanged (Fig. 2f). The developing corolla became yellowish in the morphotypes with white flower (BUR7, E-5, GTA-3 and ZHLA-1), slightly purple in purple-flowered morphotypes (BUR13, TK9-12 and ZHLA-2), and light pink in pink-flowered ones (GBO-4 and T-10). The *developed bud stage* occurred in average 61 DAS. The time from *developed* to *mature bud* (S4) stage varied from a few hours to 2 days. The S4 stage was characterized by a more pronounced pigmentation of the upper part of the standard petal (Fig. 2g). During this stage, the petals expanded rapidly until they reached their final size. The stamens grew significantly to position the anthers approximately at the same level as the stigma, the style still bent (Fig. 2j). S4 stage usually occurs 62 DAS (Table 2). The S5 stage (*open flower stage*) was defined as when the flower fully opened with sepals and petals bent backwards (Fig. 2h). The inner face of the standard petal was strongly pigmented in purple- and pink- flowered morphotypes. Stamina elongated further to position the anthers right above the stigma which has straightened up (Fig. 2k). Depending on the morphotype, the flower opened between 53 and 73 DAS with an average across morphotypes of 64 DAS. The S5 stage lasted around 1-2 days before the *flower wilted* (S6). At S6 stage, the flower started to dry and petals to shed (Fig. 2i). The stigma became brown-blackish and the anthers already drying became brown (Fig. 2l). Flowers typically wilted about 66 DAS.

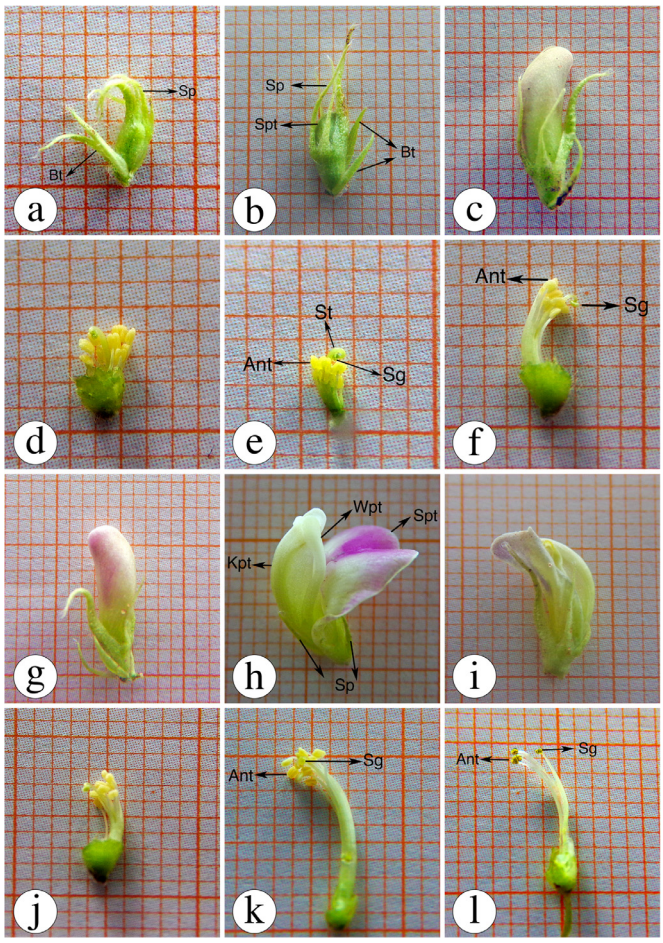

Fig. 2. Stages of floral development in Kersting's groundnut (T10 morphotype with black seed and pink flower). (a, d) - initiated flower, (b, e) - young bud, (c, f) - developed bud, (g, j) - mature bud, (h, k) - open flower, (i, l) - wilted flower. Ant - anther, Bt - bracteole, Sg - stigma, Sp – sepal, Spt – standard petal, St – style, Kpt – keel petal, Wpt – wing petal. Grid cells = 1 mm.

Table 2
Variability in the timing of six designated flower developmental stages in Kersting's groundnut.

Accession		Flower developmental stage					
		S1	S2	S3	S4	S5	S6
BUR13	*Mean ±SE*	52 ± 0.76^{c}	$59 \pm 0.80c$	60 ± 0.79^{c}	61 ± 0.81^{c}	$63 \pm 0.85c$	65 ± 0.84^{c}
	Range	34 - 67	38 - 70	39 - 72	40 - 73	41 - 84	43 - 80
	CV	16.88	15.75	15.27	15.24	15.48	15.00
BUR7	*Mean ±SE*	47 ± 0.88^{b}	53 ± 0.88^{b}	54 ± 0.87^{b}	55 ± 0.88^{b}	57 ± 0.88^{b}	60 ± 0.89^{b}
	Range	29 - 78	38 - 82	39 - 83	39 - 83	38 - 83	42 - 89
	CV	24.37	21.99	20.9	20.79	20.38	19.76
E-5	*Mean ±SE*	43 ± 1.0^{a}	49 ± 1.0^{a}	50 ± 1.0^{a}	51 ± 1.0^{a}	53 ± 1.1^{a}	55 ± 1.1^{a}
	Range	25 - 81	38 - 90	39 - 91	40 - 93	40 - 95	42 - 96
	CV	28.32	25.2	23.86	24.12	24.21	22.80
GBO-4	*Mean ±SE*	52 ± 1.2^{c}	59 ± 1.1^{c}	60 ± 1.1^{c}	61 ± 1.2^{c}	63 ± 1.2^{c}	65 ± 1.1^{c}
	Range	29 - 68	32 - 70	34 - 72	35 - 73	36 - 74	39 - 78
	CV	22.89	19.18	18.62	18.53	18.1	17.30
GTA-3	*Mean ±SE*	63 ± 1.3^{e}	70 ± 1.3^{e}	71 ± 1.3^{e}	72 ± 1.3^{e}	73 ± 1.3^{e}	76 ± 1.4^{e}
	Range	45 - 90	51 - 93	52 - 94	52 - 96	53 - 99	54 - 101
	CV	16.06	3.49	3.59	3.85	3.83	3.28
T-10	*Mean ±SE*	56 ± 0.52^{d}	64 ± 0.40^{d}	65 ± 0.39^{d}	66 ± 0.40^{d}	68 ± 0.41^{de}	70 ± 0.39^{d}
	Range	34 - 75	44 - 83	46 - 84	45 - 84	45 - 88	48 - 91
	CV	11.68	7.8	7.43	7.51	7.59	7
TK9-12	*Mean ±SE*	61 ± 0.52^{e}	67 ± 0.54^{e}	68 ± 0.54^{e}	70 ± 0.54^{e}	71 ± 0.57^{de}	73 ± 0.55^{de}
	Range	42 - 76	43 - 88	46 - 89	47 - 90	48 - 92	50 - 93
	CV	8.03	7.73	7.56	7.4	7.69	7.24
ZHLA-1	*Mean ±SE*	59 ± 0.59^{de}	66 ± 0.52^{de}	67 ± 0.54^{de}	68 ± 0.57^{de}	70 ± 0.59^{de}	73 ± 0.58^{de}
	Range	45 - 68	52 - 76	53 - 78	53 - 81	56 - 84	58 - 85
	CV	7.93	6.4	6.46	6.72	6.79	6.41
ZHLA-2	*Mean ±SE*	61 ± 0.49^{e}	68 ± 0.25^{e}	69 ± 0.25^{e}	70 ± 0.27^{e}	72 ± 0.23^{e}	74 ± 0.21^{e}
	Range	45 - 67	58 - 69	59 - 70	60 - 73	63 - 75	65 - 76
	CV	8.1	3.74	3.59	3.82	3.25	2.84

S1 – Initiated flower, S2 – young bud, S3 – developed bud, S4 – mature bud, S5 – open flower, S6 – wilted flower.
Means were rounded to the nearest integer to be consistent with 24 h observation intervals.
Means with different letters in each column are statistically different at $p<0.05$ according to Tukey's test (GLMM with negative binomial error distribution).

Results from generalized linear mixed models (GLMMs) showed statistically significant differences in the timing of all floral development stages at 0.001 probability level (Table 2). The morphotype E-5 bloomed significantly earlier (43 ± 1.0 DAS) and was also the earliest in the case of all subsequent flower developmental stages. Greater number of days to young, developed and mature bud stages were observed for the morphotypes TK9-12 and ZHLA-1. The morphotypes GTA-3 and ZHLA-2 were late in all stages (Table 2).

The flower development time (initiation to wilting) significantly differed among morphotypes (χ^2 = 30.72, df = 8, p = 0.000). It varied from 13 to 16 days with an average of 14 days across morphotypes. The morphotype with black seed and pink flower (T-10) had the longest flower development time (16±1.1 days) while the shortest time was recorded for BUR7, the morphotype with black seed and white flower.

3.2.2. Stigma receptivity, anther dehiscence and anthesis

The hydrogen peroxide test indicated that the onset of stigma receptivity (at least 50% of flowers having receptive stigma) was the first day of flower initiation, in the morning (S1 stage; Fig. 3). The stigma remained receptive for several days ranging from 6 (BUR13) to about 8 days (ZHLA2). Results of the generalized linear model (GLM) with quasibinomial error distribution showed a significant difference in stigma receptivity with respect to morphotypes (χ^2 = 29.20, df = 8, p = 0.000), and this difference was found to change over time (χ^2 = 19.04, df = 8, p = 0.015). The onset and span of anther dehiscence also varied with respect to morphotypes (χ^2 = 17.78, df = 8, p = 0.023), and time (i.e. age of flower bud) greatly impacted the effect of morphotype on anther dehiscence (χ^2 = 28.51, df = 8, p = 0.000). The onset of anther dehiscence, defined as when at least 50% of flowers have dehiscing anthers, occurred earlier in the morphotype BUR13 and ZHLA-2 (Fig. 3). The morphotypes TK9-12 and BUR7 were late to initiate anther dehiscence. Observations revealed a protogynous character of the flowers. Indeed, the stigma became receptive a few days before anthers start dehiscence. Anther dehiscence occurred at *developed bud* stage (S3) when the floral bud is still closed. Based on the analysis of onset and duration of stigma receptivity and anther dehiscence (Fig. 3), anthesis time was determined for each morphotype. Most flowers reached anthesis 3-4 days from young bud appearance and anthesis lasted from 2 to 4 days depending on the morphotype (Fig. 3). However, it was observed that a few flowers can reach anthesis earlier (2 days after young bud appearance) or later (5 days after bud appearance). The onset of anthesis coincided with the *developed bud* (S3; Fig. 2c) stage of floral development in all morphotypes and spanned to *open flower* stage (S5, Fig. 2h).

3.2.3. Fruiting phenology

Consistent to flowering phenology, six stages of fruit development were identified: beginning peg (F1), beginning pod (F2), full pod (F3), beginning seed (F4), full seed (F5), and mature seed (F6; Fig. 4). Fruit development started by the *beginning peg stage* (F1). This stage corresponded to when the gynophore elongated geotropically carrying the fertilized ovary towards the soil. The *beginning peg* stage was defined as the time when 50% of the plants developed at least one peg. It occurred 67 DAS in average and lasted 15 days. The peg elongated between 2.5 to 3.5 cm before podding began (Fig. 4a). The F2 stage, *beginning pod*, was defined as when the ovary tip started to swell and had at least twice the diameter of the peg (Fig. 4b). This happened in average 82 DAS. During this stage, the ovary gradually developed into a *full pod* (F3). The *full pod stage* (Fig. 4c) was attained 94 DAS in average. At this stage the pod had it final shape and was green irrespective of the morphotype. Cross sections revealed that pods contained only a small fertilized ovule at this stage (Fig. 4g). The F4 stage (Fig. 4d), *beginning seeding*, followed around eight days after F3 stage. Cross section observations showed that at this stage, 50% of the examined plants had at least one fruit in which the seed was sufficiently developed to show visible cotyledon sections when cut with a

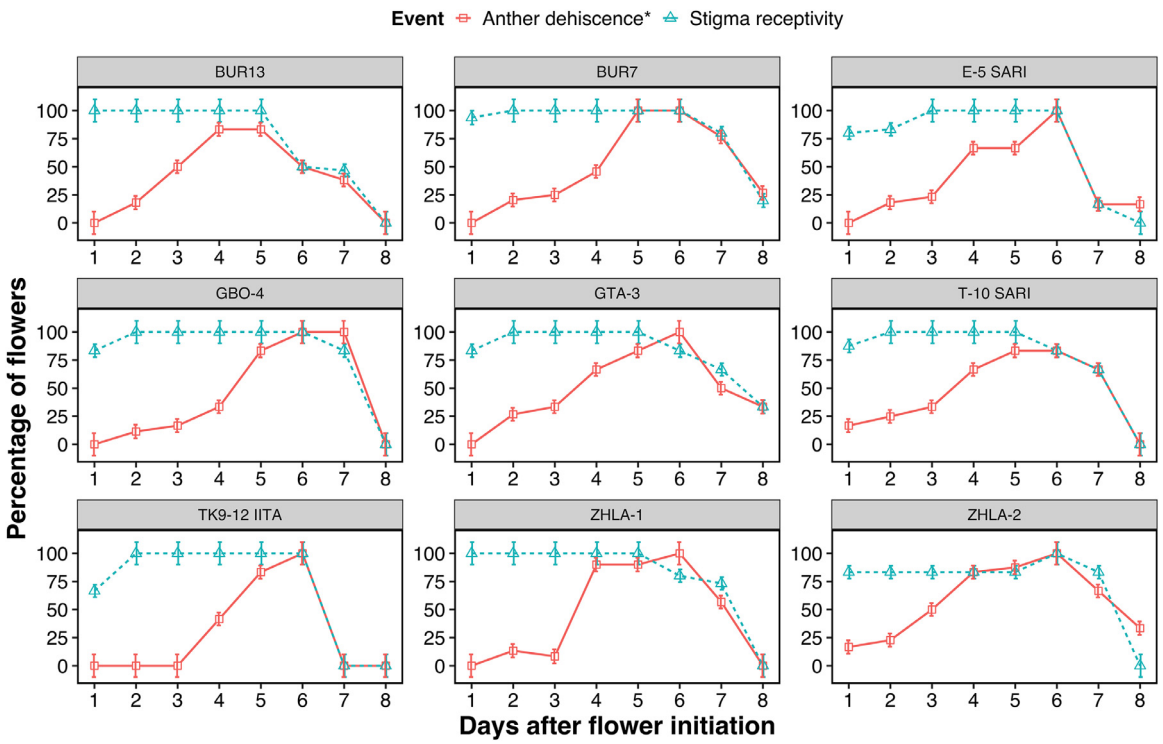

Fig. 3. Anther dehiscence and stigma receptivity patterns in 9 morphotypes of Kersting's groundnut.
* percentage of dehiscent anthers shedding pollen and/or carrying some pollen grains adhering to the anther wall.

sharp blade (Fig. 4h). The F5 stage (Fig. 4e), *full seed*, was attained when in 50% of plants, the seed appeared to fill the pod cavity. The seed contained in the pod was, however, still green indicating that it was immature (Fig. 4i). The pod was also green in all morphotypes. The F6 stage, *mature seed*, was attained when in 50% of plants, at least one pod had changed colour and became white or white tinged with purple (Fig. 4f), indicating that the seed it contained has attained physiological maturity (Fig. 4j).

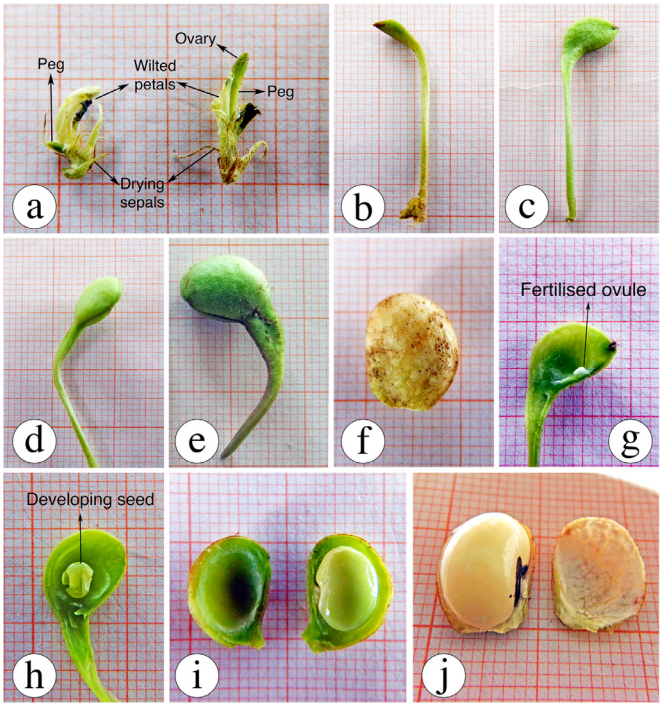

Fig. 4. Stages of fruit development in Kersting's groundnut. (a) – beginning peg, (b) – beginning pod, (c, g) – full pod, (d, h) – beginning seed, (e, i) – full seed, (f, j) – mature seed. Grid cells = 1 mm.

The timing of all fruit development stages statistically differed among morphotypes (Table 3). The morphotype E-5 initiated fruiting significantly earlier (GLMM: χ^2 = 622.03, df = 8, p<0.001). The morphotypes GTA-3, TK9-12 and ZHLA-2 were late to initiate fruiting. Table 3 also indicates that pod development (GLMM: χ^2 = 57.3, df = 8, p<0.001) and initiation of seeding (GLMM: χ^2 = 45.26, df = 8, p = 0.001) were significantly fast in E-5, BUR7 and GBO-4. Initiation of seeding, on the other hand, was late (105 DAS) in TK9-12 and ZHLA-2 (Table 3). Morphotypes were not compared for full seed (F5) and mature seed (F6) stages as only a few pods evolved into these stages, which did not allow meaningful comparison.

3.3. Pollen viability

No significant difference (GLM: likelihood ratio χ^2 = 2.74, df = 2, p = 0.253) was found in pollen viability rates among anthesis stages. Nevertheless, pollen viability significantly differed (p < 0.001) among morphotypes for each of the considered stages (Table 4). At pre-anthesis, seven morphotypes had a viability of pollen grains averaging between 94% and 97%. The minimum mean pollen viability (88%) was recorded for morphotypes GBO-4 and ZHLA-1. The highest percentage of viable pollen (97.95%) at anthesis was recorded for ZHLA-2. GBO-4 and ZHLA-1 but also BUR13 exhibited a statistically lower (GLM: likelihood ratio χ^2 = 27.43, df = 8, p = 0.001) pollen viability. At post-anthesis, GBO-4 showed the lowest pollen viability rate (82.51%) whereas the pollen viability for the other morphotypes exceeded 90% (Table 4). Fig. 5a is a microphotograph showing viable versus non-viable pollen grains.

3.4. In vitro *pollen germination and pollen growth*

There was a significant variation in pollen germination rate with respect to morphotypes (GLM: χ^2 = 181.94, df = 8, p < 0.001; Table 5). Overall, pollen germination rates were relatively low with a

Table 3
Variability in the timing of six fruit development stages in Kersting's groundnut morphotypes.

Accessions		Fruit development stages			
		F1	F2	F3	F4
BUR13	*Mean±SE*	67 ± 0.82^{c}	82 ± 0.79^{c}	95 ± 0.63^{b}	103 ± 1.1^{b}
	Range	45 - 82	59 - 102	79 - 114	84 - 132
	CV	14.29	11.27	7.57	11.62
BUR7	*Mean±SE*	61 ± 0.85^{b}	77 ± 0.85^{b}	92 ± 0.33^{a}	99 ± 0.65^{a}
	Range	44 - 89	49 - 104	77 - 113	82 - 135
	CV	18.21	14.55	4.83	8.69
E-5	*Mean±SE*	57 ± 0.99^{a}	74 ± 0.95^{a}	91 ± 0.41^{a}	100 ± 0.68^{a}
	Range	40 - 99	54 - 106	80 - 111	84 - 121
	CV	20.63	15.45	5.4	8.23
GBO-4	*Mean±SE*	67 ± 1.1^{c}	82 ± 1.1^{c}	91 ± 0.72^{a}	99 ± 1.0^{a}
	Range	44 - 81	56 - 99	63 - 109	70 - 127
	CV	16.14	12.57	7.36	9.55
GTA-3	*Mean±SE*	77 ± 1.3^{e}	91 ± 1.6^{f}	96 ± 0.93^{c}	103 ± 1.5^{b}
	Range	60 - 98	51 - 118	81 - 112	77 - 124
	CV	11.63	12.47	6.87	10.28
T-10	*Mean±SE*	72 ± 0.38^{d}	85 ± 0.48^{de}	96 ± 0.44^{c}	103 ± 0.85^{b}
	Range	53 - 91	63 - 113	75 - 113	80 - 127
	CV	6.58	7.07	5.92	10.48
TK9-12	*Mean±SE*	75 ± 0.61^{e}	89 ± 0.66^{e}	97 ± 0.61^{d}	105 ± 0.99^{d}
	Range	52 - 94	74 - 115	83 - 108	86 - 126
	CV	7.75	7.24	6.01	8.92
ZHLA-1	*Mean±SE*	74 ± 0.54^{de}	87 ± 0.58^{e}	96 ± 0.64^{c}	104 ± 1.1^{cd}
	Range	60 - 88	70 - 93	83 - 112	80 - 129
	CV	5.88	5.46	5.41	8.69
ZHLA-2	*Mean±SE*	76 ± 0.29^{e}	88 ± 0.60^{e}	97 ± 0.78^{d}	105 ± 1.3^{d}
	Range	65 - 79	74 - 104	78 - 116	81 - 136
	CV	3.89	6.81	7.76	11.38

F1 – Beginning peg, F2 – beginning pod, F3 – full pod, F4 – beginning seed. Means were rounded to the nearest integer to be consistent with 24 h observation intervals. Means with different letters in each column are statistically different based on Tukey's test (GLMM with negative binomial error distribution, $p \leq 0.001$). Morphotypes were not compared for full seed (F5) and mature seed (F6) stages as only a few of pods had reached these stages.

maximum percentage of 36.9% recorded for ZHLA-2. Lower pollen germination rates of 8.0% and 8.4% were obtained for T-10 and BUR13, respectively. All other morphotypes showed pollen germination rates ranging between 9 and 17% (Table 5). There was no significant effect of incubation time on pollen germination (GLM: $\chi^2 = 0.04$, df=2, $p = 0.979$), while a significant effect was observed for anthesis stage (GLM: $\chi^2 = 8.53$, df = 2, $p = 0.014$). Irrespective of the morphotype, pollen germination percentage was significantly higher during pre-anthesis (17.6%) compared to anthesis and post-anthesis. The interaction between incubation time and morphotype was significant indicating different responses of the morphotypes to increasing incubation time. A significant interaction (GLM: $\chi^2 = 471.56$, df = 26, $p < 0.001$) was also found between morphotype and anthesis stage revealing that the percentage of pollen germination exhibited by the different morphotypes was impacted by the anthesis stage (Table 5).

The average pollen tube length ranged from 75 μm to 215 μm (Table 5) and varied significantly with respect to the morphotype (Kruskal-Wallis: $\chi^2 = 236.07$, df = 8, $p < 0.001$). BUR7, BUR13, GBO-4 and TK9-12 had shorter pollen tubes (69.3 ± 15.5 μm to 89.1 ± 11.0 μm), while ZHLA-2 had the longest one (191 ± 4.9 μm). Pollen tube growth also differed with respect to incubation time and anthesis stage. Indeed, a significant increase in pollen tube length was noted when the incubation time was increased from 24h to 48h. However, a substantial reduction in pollen tube length was also observed after 72h of growth (Table 5). On the other hand, there was a significant reduction in pollen tube growth in response to subsequent anthesis stages. The maximum pollen growth (197.6 ± 4.5 μm) was obtained at pre-anthesis stage while the minimum growth (90.7 ± 7.3 μm) was observed at post-anthesis phase, indicating that pollen lost viability over time, irrespective of the morphotype (Table 5). Interactions of morphotype with incubation time, and anthesis stage were all highly significant ($p < 0.001$) indicating that the effect of morphotype on pollen tube growth varied according to incubation time and anthesis stage. Fig. 5b shows germinating pollen grains and pollen tubes after 24 h of incubation.

4. Discussion

Understanding floral and reproductive biology creates an avenue for genetic research and crop improvement (Kumari and Sharma 2017). In the present study, we described the reproductive organs with their phenological events, assessed stigma receptivity and anther dehiscence, and examined pollen viability and germination in nine morphotypes of Kersting's groundnut. The study is of prime importance as it provides consistent information to help breeders devise a breeding program for the crop.

Table 4
Pollen viability rates of Kersting's groundnut morphotypes before, during and after anthesis.

Accession	Pre-anthesis†		Anthesis		Post-anthesis‡	
	Mean ± SE	CV	Mean ± SE	CV	Mean ± SE	CV
BUR13	94.73 ± 1.42^{b} (69.35 - 100)	10.03	89.54 ± 3.74^{a} (19.61 - 100)	28.03	93.57 ± 1.52^{b} (66.67 - 100)	10.92
BUR7	94.18 ± 0.92^{b} (69.35 - 100)	6.58	93.31 ± 0.92^{ab} (80.14 - 100)	6.61	92.05 ± 0.88^{b} (80.14 - 100)	6.45
E-5	95.72 ± 0.84^{b} (69.35 - 100)	5.89	94.84 ± 1.56^{ab} (74.19 - 100)	11.01	97.13 ± 1.22^{b} (74.19 - 100)	8.45
GBO-4	88.18 ± 2.06^{a} (42.31 - 98.55)	15.67	88.33 ± 2.12^{a} (42.31 - 98.55)	16.13	82.51 ± 3.15^{a} (42.31 - 98.55)	25.58
GTA-3	94.98 ± 0.75^{b} (85.51 - 100)	5.31	96.09 ± 0.77^{b} (83.67 - 100)	5.38	92.2 ± 1.48^{b} (67.74 - 100)	10.76
T-10	95.76 ± 0.41^{b} (92.31 - 100)	2.88	93.66 ± 2.04^{ab} (19.61 - 100)	13.79	90.45 ± 2.02^{b} (62.34 - 100)	15.82
TK9-12	97.00 ± 0.61^{b} (89.39 - 100)	4.20	96.12 ± 0.44^{b} (90.79 - 100)	3.08	96.72 ± 0.56^{b} (88.89 - 100)	3.87
ZHLA-1	88.65 ± 2.05^{a} (61.11 - 100)	15.51	90.36 ± 1.23^{a} (75.00 - 100)	9.16	95.6 ± 0.60^{b} (86.49 - 100)	4.18
ZHLA-2	95.69 ± 1.22^{b} (73.13 - 100)	8.55	97.95 ± 0.36^{b} (94.37 - 100)	2.45	93.44 ± 1.51^{b} (68.75 - 100)	10.82
F-value	6.20***		3.43***		6.98***	

† One day before anthesis (young bud stage),
‡ one day after anthesis (open flower stage). Means with different letters in each column are significantly different at 0.05 probability level according to Tukey's multiple comparison post hoc test.
*** Significant at $p < 0.001$. Values between brackets are ranges (min – max).

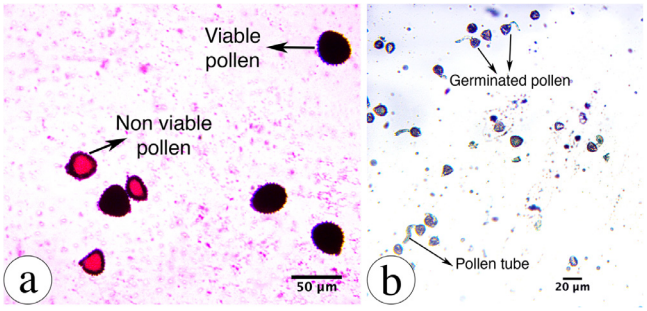

Fig. 5. Pollen viability and germinability. **(a)** Pollen viability in 1% Carbol Fuchsin, **(b)** Pollen germination after 24h of incubation at 30°C.

4.1. Floral traits related to the breeding system

The detailed observation of floral structures revealed that Kersting's groundnut presents a zygomorphic corolla with imbricate lobes, the uppermost (adaxial) lobe overlapping the others in the bud and the lowermost (abaxial) pair of lobes forming a carina that encloses reproductive organs. These are particular flower features of most plant species of the Papilionoideae subfamily of legumes (Tucker 2003; Singh 2019) which facilitate their pollination by visiting insects (Aronne et al. 2012). Another particular trait of Kersting's groundnut flowers is the presence of two parallel glandular appendages on the internal face of the vexillum. This is a specific floral trait of species belonging to the genus *Macrotyloma* (Verdcourt 1970) and was among the traits used by Maréchal and Baudet (1977) to transfer the monospecific genus *Kerstingiella* including Kersting's groundnut, then named *Kerstingiella geocarpa,* to *Macrotyloma*. However, these authors did not mention the function and role of these intriguing glands in the reproduction of the species, albeit their position on the standard petal suggests that they could be nectaries. According to Vogel (1997), many diadelphous taxa of the subfamily Papilionoideae commonly feature two nectaries that accumulate nectar at the basis of the vexillary petal, where there are usually two openings (Bernardello 2007). In any case, flower visitors seem to be rare and are unknown in Kersting's groundnut which is congruent with previous observations from Amuti (1980). Further research is needed to investigate the anatomy and morphology of these secretory structures as well as their functional role in pollination of Kersting's groundnut.

4.2. Phenology

The sequence of events occurring in the process of flower development has been widely studied in many orphan legumes (Kar and Datta 2017; Kalve and Tadege 2017; Dhanaraj 2018), unlike in Kersting's groundnut. In the present study, we identified six stages of floral development (S1 – S6) and six stages of fruit development (F1 – F6). These reproductive stages are similar, apart from a few differences, to those developed for groundnut (*Arachis hypogaea* L.) (Boote 1982) and Bambara groundnut (*Vigna subterranean*) (Dhanaraj 2018), two other cultivated subterranean legumes. Dhanaraj (2018) recently depicted the flower development scale of Bambara groundnut into six stages. The main difference between Kersting's groundnut and Bambara groundnut scales is that the S1 stage (initiated flower) identified in the former corresponds to stages 1 to 3 of the latter. Besides, the scale developed in the present study includes *wilted flower* (S6, Fig. 2i) which is absent from Dhanaraj's work. In addition, no '*over-mature pods*' stage was included here, contrary to groundnut, though the flower development stages presented here are more informative than in groundnut (Boote 1982). Indeed, our first five stages (S1 – S5) correspond to the R1 stage of groundnut. The most remarkable stage of the

Table 5
Effects of morphotype, incubation time and anthesis stage on pollen germination rate and pollen tube length in Kersting's groundnut. The bottom panel contains P-values from the GLM (pollen germination) and Kruskal-Wallis test (pollen tube length).

	Pollen germination (%)		Pollen tube growth (μm)	
	Mean ± SE	Range	Median ± SE	Range
Morphotype				
BUR13	8.42 ± 0.84^{a}	1.85-18.18	69.30 ± 15.48^{a}	30.43-166.97
BUR7	9.28 ± 0.89^{ab}	1.23-14.89	89.10 ± 10.95^{a}	20.12-258.79
E-5	11.01 ± 1.91^{abc}	3.90-37.50	105.00 ± 12.64^{ab}	26.93-322.79
GBO-4	10.23 ± 1.00^{ab}	2.86-19.23	73.70 ± 14.48^{a}	20.05-203.03
GTA-3	17.78 ± 2.24^{c}	5.88-39.13	151.00 ± 8.94^{c}	20.88-487.67
T-10	8.02 ± 1.51^{a}	1.41-27.54	145.00 ± 9.27^{cd}	37.54-403.01
TK9-12	16.04 ± 1.53^{bc}	5.45-33.33	60.70 ± 12.64^{a}	2.15-206.55
ZHLA-1	12.12 ± 1.70^{abc}	1.47-28.57	147.00 ± 9.05^{bc}	10.46-363.14
ZHLA-2	36.87 ± 3.00^{d}	13.33-74.07	191.00 ± 4.90^{d}	26.05-612.67
Time				
24H	14.41 ± 0.97^{a}	4.65-40.00	141.00 ± 5.46^{b}	2.15-436.56
48H	14.62 ± 1.75^{a}	1.23-74.07	154.00 ± 5.23^{b}	20.88-540.71
72H	14.22 ± 1.31^{a}	1.41-39.13	107.00 ± 6.97^{a}	20.12-404.56
Anthesis stage				
Pre-Anthesis†	17.56 ± 1.80^{b}	1.47-74.07	179.00 ± 4.52^{c}	2.15-404.56
Anthesis	12.30 ± 1.19^{a}	1.23-40.00	125.00 ± 5.90^{b}	2.15-335.42
Post-Anthesis‡	13.39 ± 0.95^{a}	2.86-37.50	90.70 ± 7.33^{a}	20.05-612.67
P value				
Morphotype (M)	<0.001***		<0.001***	
Time (T)	0.979 ns		0.002**	
Stage (S)	0.014*		<0.001***	
M × T	<0.001***		<0.001***	
M × S	<0.001***		<0.001***	

Means with same letters are not significantly different according to Tukey's post hoc test at $P<0.05$
† One day before anthesis (young bud stage),
‡ one day after anthesis (open flower stage)
ns not significant,
* significant at $p < 0.05$,
*** significant at $p < 0.001$

reproductive growth of Kersting's groundnut is the development of pegs (F1 stage, Fig. 4a). According to Chandra et al. (2019), it is a unique evolutionary adaptative organ that mediates the reproduction of the crop by protecting the pods from unfavourable biotic and abiotic factors of the growing environment. Unlike in groundnut where failure of peg penetration in the soil induces embryo abortion and therefore yield loss (Chandra et al. 2019), Kersting's groundnut develops pods even with pegs that have not penetrated into the soil. Nevertheless, pods that developed above the soil surface appear undersized as the subterranean peg can absorb nutrient and moisture from soil to ensure an optimal development of the pod (Chandra et al. 2019). Pod development above soil surface may hence potentially affect Kersting's groundnut yield although the magnitude of this effect is yet to be established.

4.3. Incomplete protogyny and spontaneous self-pollination in Kersting's groundnut: implications for the breeding system of the crop

We observed that Kersting's groundnut flowers are incompletely protogynous as their stigma becomes receptive 1-2 days before anther dehiscence and remains so for 4-6 days. Protogyny primarily prevents self-pollination through the temporal reduction of pollen-pistil interferences in flowering plants (Fernández-Illescas et al. 2011; Griffin et al. 2000). However, protogyny is not always effective in preventing or even reducing selfing (Friedman and Barrett 2009). Despite their protogynous nature, some species can force self-pollination using sometimes unusual mechanisms (Zhang and Li 2008; Freitas and Sazima 2009). In the case of Kersting's groundnut, the flowers are found to be 'pre-anthesis cleistogamous' as described by Lord (1981). This means that bud pollination occurs before anthesis, which should lead to a high rate of selfing in the crop. On the other hand, the phenological events of flowering revealed style movement towards the anthers at stages S2 and S3, suggesting a spontaneous self-pollination as reported earlier in *Tephrosia purpurea* (Kumari and Sharma 2017), *Calolisianthus pendulus* and *Deianira nervosa* (Freitas and Sazima 2009). Evidence revealed that style or stigma movements favour selfing in various plant families including Lamiaceae (Ganie et al. 2015), Leguminosae (Kumari and Sharma 2017), Malvaceae (Wani et al. 2015), Valerianaceae (Khajuria et al. 2011), Violaceae (Culley 2002), and Zingiberaceae (Li et al. 2002; Zhang et al. 2003). In Kersting's groundnut, the incomplete protogyny coupled with a style movement exposing the already receptive stigma to the anther seem to induce the spontaneous self-pollination at an early flowering stage. The same mechanism is observed in the Papilionoideae legume weed *T. purpurea*, which exhibits an obligate autogamy (Kumari and Sharma 2017). However, the mechanism observed here is slightly different than in *T. purpurea*. In fact, the standard petal of Kersting's groundnut forms two small glands (Fig. 1d) located at the same level with the stigma and the anthers, which exude a sticky substance that seems to stick the pollen grains to the stigma surface. An obligate autogamy in Kersting's groundnut may facilitate the maintenance of homogeneity of parental lines in breeding programs. Conversely, it may also have detrimental consequences such as reduced genomic variation (Lam et al. 2010), increased linkage disequilibrium and inbreeding depression (Wright et al. 2013). This corroborates the finding from Pasquet et al. (2002) who studied the genetic diversity in the crop using isozymes and revealed an extremely low level of genetic variation in the species. Further studies relating to the investigation of potential self-incompatibilities are necessary to validate the assumption of strict autogamous selfing in Kersting's groundnut.

4.4. Anthesis timing and its implication for hybridization

Identifying the appropriate time for emasculation is critical for breeders as it increases chances for successful crossing. Based on the finding obtained from the evaluation of anther dehiscence and the spontaneous self-pollination observed in the crop, this study recommends that emasculation be performed early on young flower buds one day old. Such buds are generally 5-6 mm in length (Fig. 2b). Early emasculation will hence prevent unwanted self-pollination since it is found that buds greater than this size may have been spontaneously self-pollinated. Emasculation of 1-day old buds would, however, be challenging as Kersting's groundnut young buds are particularly fragile and difficult to manipulate due to their small size, twisted keels, and basal position. The same constraints added to a high rate of abortion following mechanical manipulation of floral organs were also reported to limit successful hybridization in Bambara groundnut (Azam-Ali et al. 2004). Pollen to be used for hybridization should ideally be sourced from mature or freshly opened buds (i.e. 3 to 4 days old buds). As illustrated in other orphan legume crops including Bambara groundnut (Azam-Ali et al. 2004) and chickpea (Kalve and Tadege 2017), the crossing technique is also decisive in the success rate. Thus, it may be necessary to assess the efficiency of different techniques and optimize a crossing protocol for this neglected crop to foster its genetic improvement in Benin and West Africa. This study provides the basis for choice of strategies and periods to operate in order to develop such protocol.

4.5. Pollen viability, germinability and pollen tube development

Pollen grains exhibited a great viability level which is maintained up to 24 h after anthesis. Thus, pollen grains can potentially be conserved for a few days to few weeks in appropriate conditions. This capability would be a major advancement for hybrid development in Kersting's groundnut. However, some authors (Veiga et al. 2012; Soares et al. 2008; Rathod et al. 2018; de Souza et al. 2017) argued that, though histochemical viability tests are simple, straightforward and cheap techniques, they tend to overestimate pollen viability and would, therefore, provide misleading information regarding pollen viability. This is consistent with our findings where percentages of pollen viability above 82% were observed as revealed by the Carbol fuchsin staining, whereas the average *in vitro* germination rates only ranged from 8 to 36% (Table 5). However, the low pollen germination rate observed may be the result of *in vitro* germination, which according to Galletta (1983), often underestimates pollen viability. To bypass the bias that could be introduced in estimation of pollen viability through *in vitro* germination test, authors recommend to correlate pollen germination rates with production traits such as fruit and seed yield (Soares et al. 2013; Valadares et al. 2019). Therefore, investigating the correlation between yield and pollen *in vitro* germination may be a worthy research objective in future works.

We found that accession ZHLA-2 which has cream seed and purple flower exhibited the highest pollen germination rate and pollen tube development. Hence, the male gametes of this morphotype have a high vigour, making it a potentially good candidate as a pollen donor for hybridization. Nevertheless, the choice of paternal and maternal parents predominantly depends on the breeding objectives. The unexpected highest pollen germinability and pollen tube growth recorded for pre-anthesis pollen grains can be explained by the fact that pollen grains having matured newly were still more viable compared to post-anthesis where grains were aging, and then losing viability. On the other hand, the effect of incubation time on pollen germination was not significant suggesting that pollen germinated very quickly and attained a maximum germination rate in only a few hours. This pattern is consistent with a study on *Swainsona formosa* (G.Don) J.Thompson, a Papilionoideae species whose pollen presented maximum germination after only 1 hour of incubation (Zulkarnain 2019). Therefore, shorter time intervals should be considered in future pollen germination studies.

Author contributions statement

KMK, EEA, GHD and ACA designed the study. KK conducted the experiments analysed the data, with technical support from EEA, GHD and ACA. SA actively participated in data collection. KMK developed the manuscript. CAG provided lab facilities and technical support for *in vitro* pollen germination study. HSS supported lay out of experiments and manuscript development. HY, CA, and AEA provided guidance throughout experiments, data management and manuscript development. All authors reviewed, improved the manuscript and agree to be accountable for the final manuscript.

Funding

This work was supported by the Netherlands Organisation for Scientific Research (NWO-WOTRO) [grant numbers W08.270.344] and the Regional Universities Forum for Capacity Building in Agriculture (RUFORUM) [grant number RU/2018/TQA/38].

Declaration of Competing Interest

None

Acknowledgments

We thank Dr. Arnaud Agbidinoukoun and Mr. Jacques Aissi from the Central Laboratory of Plant Biotechnology and Plant Breeding, Faculty of Sciences and Techniques, for assistance during *in vitro* pollen germination study. We are also grateful to Mr. Ismaïla Orou Bah for drawing the floral diagram.

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