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Phenology, floral organs ultra-structure, traits correlation, stigma receptiveness, pollen viability and germinability in horsegram (*Macrotyloma uniflorum* Lam.) Verdc.

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A B S T R A C T

The absence of information on the reproductive biology of some nutritive legumes has been an obstacle to their genetic improvement. Gathering information on reproductive biology contributes significantly to the interest of breeders to have adequate tools for initiating the improvement program through crosses among high performing genotypes. To contribute to this, we examined the flowering phenology, pollen biology, stigma receptiveness at bud dehiscence and anthesis phases, and the in-vitro germination of the pollens in horsegram (*Macrotyloma uniflorum*). Pollens of horsegram were found to be either tricolporate or triporate, echinates in all accessions except in the collections from Benin. Ovaries in horsegram showed great variation in the number of ovules and activities of peroxidase indicated the receptiveness of stigma at bud dehiscence phase and during anthesis. A high number of ovules per ovary was found in accessions from Australia (Leichardt 1 and 2), Mali (LeichardtMali) and India (CPI22679). Pollen viability analysis implies the possibility of better results when pollinating during the day and immediately after the flower opens. The study provides information on the reproductive biology of horsegram for future hybridization program in order to enhance the performance of the crop and increase production. It also reveals differences in accessions from Benin compared with all other accessions from Mali, India or Australia.

1. Introduction

The legume family (Fabaceae) contain 1300 species and is tagged as the second most important food crop family. Legumes are abundantly rich in proteins and bioactive compounds necessary for the proper daily intake of nutrients. Many of them are orphan and propagated by smallholder farmers who use them as a source of income and nutrients (Prasad and Singh, 2015). Orphan or underutilized legume crops are staple food crops in most developing countries, but their economic importance in global markets is limited. They also attracts inadequate attention from development organizations and are mostly cultivated in marginal areas for local economy and consumption. Furthermore, those crops are also poor attractors of the global research network (Cullis and Kunert, 2017). Recent effects of climate change on major crops has driven the attention of scientists towards minor species due to the high tolerance to most of the biotic and abiotic stresses (Bhartiya et al., 2015).

Legumes with global production and consumption quantities, include common bean (*Phaseolus vulgaris* L.), chickpea (Cicer arietinum L.), dry pea (*Pisum sativum* L.), lentil (Lens culinaris Medik.), broad bean (*Vicia faba* L.), soybean (*Glycine max* L.), pigeon pea (*Cajanus cajan* L. Millsp.), and cowpea (*Vigna unguiculata* (L.) Walp). Orphan legumes with little attention from the mainstream research network include for instance, moth bean (*V. aconitifolia* L.), dolichos (Lablab purpureus (L.) Sweet.), Marama bean (*Tylosema esculentum* (Burch.) A. Schreib), black bean (Vigna mungo (L.) Hepper), Kersting's groundnut (*Macrotyloma geocarpum* (Harms) Maréchal and Baudet), Bambara groundnut (Vigna subterranea (L.) Verdc.), and horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.). Horsegram was cultivated for

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food and was mainly used as fodders in feeding horses in the prehistoric era of Asia and African continents (Aditya et al., 2019). Harvested area and production statistics are poorly documented around the world. However, India is regarded as the center of production of horsegram whereas Africa remain the gene-rich region for this species due to the availability of large number of wild species of horsegram and other species in the genus *Macrotyloma* (Aditya et al., 2019). Horsegram exhibits notable levels of minerals and has excellent antioxidant characteristics that are capable of neutralizing free radicals, which is crucial in the fight against chronic diseases (Morris, 2008; Tsamo et al., 2020). Horsegram has been used for its therapeutic properties in addressing a range of health conditions, such as the common cold, fevers, renal calculi, leucorrhoea, vascular disorders, and constipation (Choudhary et al., 2022). Lectin-rich protein and myricetin content in horsegram showed an anti-hyperglycemic effect, which improved diabetic conditions by reducing elevated sugar levels in induced rats (Aditya et al., 2019; Lalitha et al., 2020; Tiwari et al., 2013).

Horsegram exhibits wider adaptability with tolerance to several biotic and abiotic stresses. The crop thrives well in dry land as several reports has confirmed their resistance to drought. Several genes characterized as drought resistance genes were identified in horsegram which place the species as an important crop in breeding for drought tolerance (Choudhary et al., 2022; Reddy et al., 2008).

The crop is fifth mostly grown legume in India for consumption and also for folders use in feeding animals. The yield is very low even in India which is less than 500 kg.ha^{-1} as compared to other legumes such as pigeon pea, chickpea and Lentil (Aditya et al., 2019; Fuller and Murphy, 2018). Improved varieties with resistance to stresses would speed up productivity in regions where this crop is already consumed, to sustain crops diversification.

Already identified accessions of horsegram was stated clearly to be facing improvement challenges which include very low yield, small seeds size, and absence of desirable traits (Chahota et al., 2020). Owing to this, breeding for desirable traits would be feasible through proper knowledge of the reproductive biology of diverse accessions of horsegram from the different eco-geographical regions.

The knowledge of floral biology is a prerequisite for any hybridization and genetic improvement of crops through conventional breeding (Douglas and Freyre, 2010; Kaur et al., 2011). Understanding the pollen biology and stigma receptivity phases is useful to successful crossing of genotypes (Gan et al., 2013). The recent success in the pollen and stigma compatibility study of some underexploited legumes such as Bambara groundnut (*Vigna subterranea* (L.) Verdc.) (Chandra et al., 2017), Pigeon pea [*Cajanus cajan* (L.) Millspaugh] (Kar and Datta, 2017) and Kersting's groundnut (*Macrotyloma geocarpum*) (Kafoutchoni et al., 2020) have contributed to the recent interest of breeders in the improvement of these species.

Despite the potential of horsegram, little is known about its floral biology. According to the available information, there is scanty knowledge of floral biology in this crop. In brief, a study on the variability level of floral attributes of horsegram was reported (Bhave and Dhonukshe, 1991); however, floral development phases, pollen biology and stigma receptive remain less documented. Consequently, more investigations are needed to characterize the diverse collections of horsegram to reveal the phases involved in floral development of it, unravel the pollen viability level at different time, stigma receptivity, variations in ultra-structures of the pollen, pollen in-vitro germination and the number of ovules per ovary which will pave the way for the initiation of proper hybridization pattern.

Horsegram is papilionaceous, hermaphroditic, and zygomorphic. The calyx has five sepals and is gamosepalous. The calyx is short and grows in size till anthesis. As the flower opens, its size reduces. The corolla, on the other hand, is polypetalous with five petals. The horsegram flower has a big and broad standard that covers the rest of the flower parts. The androecium of the flower is made up of 10 stamens that are diadelphous in nature which surround the stigma to enable self-pollination (Bhave and Dhonukshe, 1991).

The present study intends to unlock knowledge on floral biology to contribute to harnessing the breeding efforts efficiently. It depicts the variation among the accessions of horsegram based on the floral traits. Therefore, we investigated the pollen pistil compatibility range and analyze the correlation between the floral traits. The study addressed the following research questions: are there variation among horsegram accessions with respect to floral traits? Is there any significant correlation among the pollen and ovary structure within species? Can floral traits be used in characterizing the accessions of horsegram? To contribute to these research questions, we hypothesized that (i) there are significant variations among *M. uniflorum* accessions in terms of floral structure and reproductive architecture; (ii) stigma receptiveness and pollen viability occur only at anthesis stage.

2. Materials and methods

2.1. Pant materials and experimental design

Twenty-five (25) accessions (Table 1) of horsegram from Australia, Asia, and Africa were collected from the genebank of the Genetics, Biotechnology and Seed Sciences Unit (GBioS), at the Faculty of Agricultural Sciences, University of Abomey-Calavi, Republic of Benin. Two experiments were implemented in Nigeria and Benin from March to September 2020. The first experiment was carried out in Benin at the Genetics, Biotechnology and seed sciences Unit (6° 25′00.8″N, and 2°20′24.5″E) in Abomey-Calavi. The Abomey-Calavi site has a bimodal rainfall with an annual rainfall and average temperature of 1000 mm and 27 °C respectively (Houdegbe et al., 2018). The second experiment was conducted at the experimental farm of the Faculty of Agriculture and Natural Resources Management, Ebonyi State University (EBSU), Abakaliki, in the South-eastern region of Nigeria (6°151 N latitude and 8°100E longitude). The Abakaliki site

Table 1
List of accessions of horsegram (*Macrotyloma uniflorum*) and their country of origin.

Accession code	Origin	Source
AKE01	Benin	GBioS
AKE02	Benin	GBioS
AKE04	Benin	GBioS
AKE05	Benin	GBioS
AKE07	Benin	GBioS
AKE08	Benin	GBioS
AKE09	Benin	GBioS
AKE10	Benin	GBioS
AKE11	Benin	GBioS
AKE12	Benin	GBioS
AKE13	Benin	GBioS
AKE14	Benin	GBioS
AKE15	Benin	GBioS
AKE16	Benin	GBioS
AKE17	Benin	GBioS
AKE18	Benin	GBioS
AKE19	Benin	GBioS
AKE20	Benin	GBioS
LIECHARDT1	Australia	GBioS
LEICHARDT2	Australia	GBioS
LIECHARDTMALI	Mali	GBioS
CPI52592A	Tanzania	GBioS
CPI52592B	Tanzania	GBioS
HORSEGRAM250E	Nepal	GBioS
CPI22697	India	GBioS

*GBioS: Genetics, Biotechnology and Seed Science Unit, Faculty of Agronomic Sciences, University of Abomey-Calavi, Republic of Benin.

has a bimodal rainfall with the main rainfall pattern occurring from April to October, with its first peak often occurring in July and the second in September with an annual rainfall and average temperature of 1100 mm and 26.7 °C respectively (Njoku et al., 2017).

Prior to the experiments, physical and chemical properties of soil samples from 0 to 20 cm depth were analyzed at the Laboratory of Soil Science at the Faculty of Agronomic Sciences, University of Abomey-Calavi and at the Laboratory of Biochemistry of Alex Ekweme Federal University, Nigeria respectively for the GBioS experimental site and the Campus of Agricultural Sciences (CAS) of the Ebonyi State University (EBSU) experimental site. The CAS-EBSU experimental site was characterized by hydromorphic soil with sandy loam texture whereas the experimental site of GBioS was characterized by ferralitic soil type (Volkoff and Willaime, 1976) with sandy loam texture.

The soil of the two experimental sites were slightly acid with good permeability and drainage. The soil of the site in Benin contained 23.06 $mg.kg^{-1}$ of available phosphorus, 811.2 $mg.kg^{-1}$ of available potassium, 287.95 $mg.kg^{-1}$ of magnesium and 126 $mg.kg^{-1}$ of calcium (Houdegbe et al., 2018). The soil of CAS, in Ebonyi State University contain 28.4 mg.kg-1 of available phosphorus, 84.06 mg.kg-1 of potassium; 680.68 mg.kg-1 of Magnesium, 1523.04 mg.kg-1 of calcium.

Incomplete block design (alpha lattice) with three replicates was laid out at these two locations. Each experiment plot consisted in a 1.5 m long row with 0.3 m spacing between plants; the distance between plots was 0.8 m and the distance between replicates was 1 m. Each plot had 6 plants giving a total of 18 plants per accession and per replicate. The randomization of the design was done using R package “Agricolae” with an efficiency factor of 0.75. The descriptors list used in this study was developed in 2015 by the World Vegetable center for *Macrotyloma* species (AVGRIS, 2015). The floral data was assessed on fully opened flowers of all accessions considered. Flowers were randomly collected and dissected to describe different parts of the flower. We measured the stamen length, pollen diameter, ovary length and width, sepal length and width, standard length and width, peduncle length, bud length and considered the maturity trait (days to flowering).

2.2. Data collection

Five hundred flowers (with a minimum of 20 flowers per accession) were selected and tagged for studying the in-depth floral developmental phases of horsegram and clear pictures were taken. The data collection was initiated from sowing to the budding stage and finally till after flower senescence ended. Quantitative traits such as length and width of standard, length of the stamen, pistil length and width of calyx, length and width of the ovary were collected. Fresh flowers were also collected from all accessions at a different time range of 7 h AM, 11 h AM, 15 h PM and 19 h PM for pollen viability analysis applying the method of Rathod et al. (2018) through the use of 2% acetocarmine solution (Sigma Aldrich). In-vitro germination was also assessed through the use of 10% sucrose solution (Rathod et al., 2018). Variation in ultra-structures of pollen was evaluated in the palynology unit of the Laboratory of Botany and Plant Ecology where the flower samples of horsegram were collected and dissected with a magnifying glass to look for pollen grains. Once mounted between slides and coverslips, they were observed and described using the Jeulin®□ light microscope with integrated camera at X40O and X1000 magnifications. The characters considered were symmetry, shape, size, apertures (pores or furrows) and ornamentation of the exine.

Stigma receptivity was carried out at bud dehiscence and anthesis stages using the modified method of Monika and Parihar (2017). The dark-red coloured grains of pollen were counted as viable pollens while unstained ones were counted as non-viable or aborted pollens. The pollen viability rate was calculated using Rathod et al. (2018) with:

$$\textit{Pollen viability}\ (\%) = 100\ (\textit{Number of stained pollen grains}/\ \textit{Total number of pollen grains on slide})$$

The maturity data, days to flowering was also recorded accurately. Stigma receptivity was examined in horsegram following the modified method of Monika and Parihar (2017). Pollens with tube longer than the diameter of the pollen are considered as germinated pollens (Gupta et al., 2017). Ultra-structures of pollen were assessed when pollen was collected from fresh flower, placed on a clean cavity slide. Glycerine jelly was prepared and added to the slide. The slides were heated for a few seconds and covered with a slip. The slide was then sealed with jelly and then heated for some seconds before viewed under a light microscope using X100 with immersion oil. Also, variability in the number of ovules per ovary was observed. The ovary of all accessions evaluated in this study were dissected longitudinally using scalpels and dissecting microscope. Ovules number were counted for each accession.

2.3. Statistical analysis

The data collected was subjected to analysis using R software version 3.6.3 (R Core Team, 2019) and Sigma plot version (12.0). Chi-square test for independence was carried out to analyze the variation among the twenty-five accessions of horsegram based on floral and other morphological traits. Data were subjected to the Kruskal Wallis test to ascertain whether the difference observed among the accessions was significant ($p \leq 0.05$) for various traits. The coefficient of variation was analyzed for all the traits using CV (%) = x/σ * 100 (Kozak et al., 2013). A correlation test was performed to detect the relationship among the quantitative floral variables. Boxplot was used to illustrate the variation of floral traits across the origin of the accessions. Bar plots were adopted to differentiate the performance of the group of accessions with similar pollen type.

3. Result

3.1. Phenology, pollen viability and ultrastructure

Regardless of the accessions’ origin, the developmental phases of the floral organ of horsegram have been grouped into four phases. The first stage involved the initiation of the primordial bud. This stage opens the development of the reproductive organ of this species. The primordial bud protrudes after the range days of 33 to 35 from sowing (Fig. 1A and 1E). The structure developed into full bud after four days of the initiation. At the budding phase, the petal is distinctly shown, having the standard as white in accessions from Benin and yellow in accessions from other countries. Wings and keels were enfolded and remain compacted with the androecium and gynoecium; the flower bud remains in an enclosed form prior to the inception of bud dehiscence (Fig. 1B and 1F). The third stage (anthesis) showed the opening of the flower. This stage showed the distinct separation of the petal (standard, wings and keels), pubescent sepal in all accessions evaluated (Fig. 1C and 1G). Stigma is highly receptive and the pollen revealed a high degree of viability. This stage lasted for three days before proceeding to the phase where there is degeneration of the perianth (senescence). This phase involved the degeneration and decolouration of the petals (white to orange in accessions from Benin and yellow to orange in other accessions) (Fig. 1D and 1H). The petal color is white in all accessions from Benin (Fig. 1C) whereas accessions from Australia, India, Nepal and Tanzania and Mali exhibits greenish-yellow flower with a purple dot on standard (Fig. 1G). Besides the variation pointed, the arrangement pattern exhibited a high degree of similarities across all Horsegram

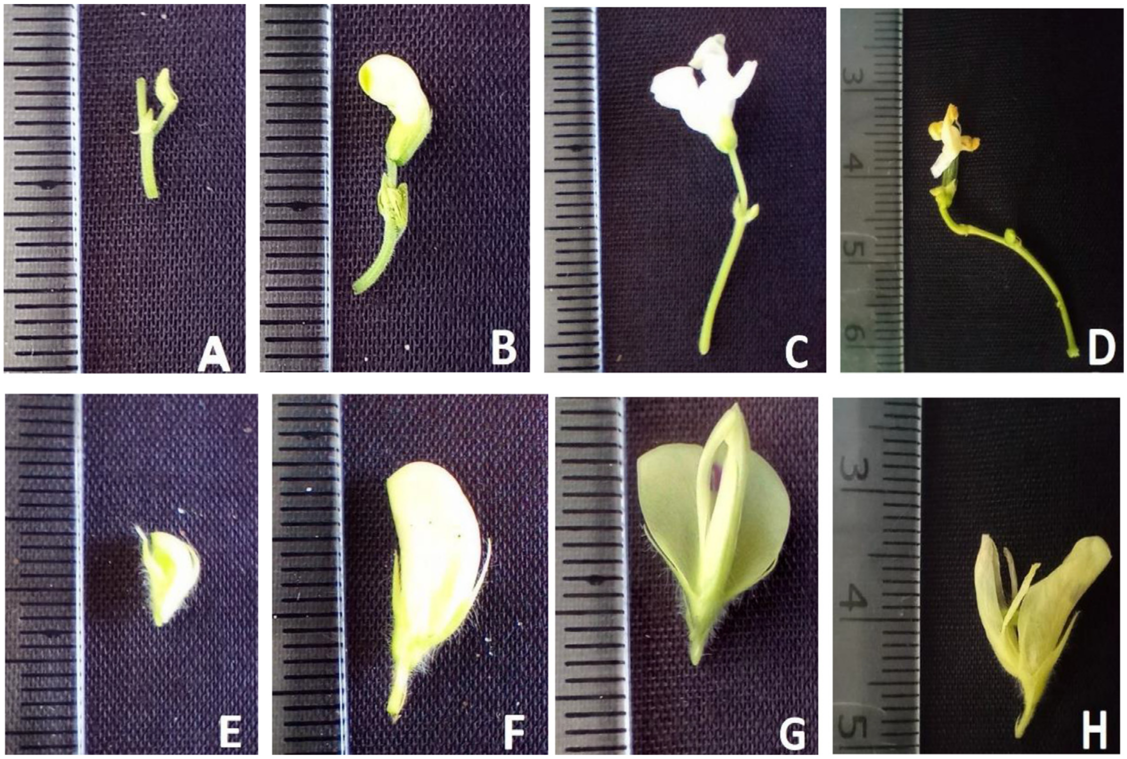

Fig. 1. Flower developmental stages in horsegram (*Macrotyloma uniflorum*); accessions from Republic of Benin (A to D) and from other countries (E to H). **A/E**- Primordial/Initiation of bud, **B/F**-Budding/prior Dehiscence, C/G- Anthesis, D/H-Senescence. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

accessions. All horsegram reproductive organ exhibits estivation which refers to the arrangement of different parts of the flower. The innermost part of the petal is very small and the outermost part is large having a structure called standard. Petals are arranged into three distinct patterns having a standard (Fig. 2A and 2E), two wings (Fig. 2B and 2F) and two keels (Fig. 2C and 2G).

The size of the standard varies across the accessions; the wings cover the keels that houses the anther and the stigma. The wings and keels are of smaller sizes compare to a broad, large standard clearly shown in Figs. 1 and 2. The keels serve as a protector to the anther. Easy separation of these keels can be done in accessions sourced from all countries except accessions from Benin whose keels could result in the breaking of the androecium structures due to the coiled pattern of the keels around the androecium. The flower exhibit determinate flowering (plants flowers till they fully wilted).

3.2. Variability of floral traits in Horsegram

Pollen diameter varied from 30.50 μm to 68.0 μm with an average of 43.78 $\pm$ 8.65 μm. The mean value of stamen length was 8.07 $\pm$ 0.83 mm; it varied from 4.78 mm to 9.14 mm. Peduncle length mean value was 16.30 $\pm$ 7.55 mm; it varied from 6.01 mm to 34.63 mm. Bud length varied from 4.64 mm to 14.53 mm with an average value of 7.50 $\pm$ 3.26 mm. Ovary length ranged from 4.10 mm to 8.75 mm with an average value of 5.80 $\pm$ 1.17 mm; ovary width varied from 0.58 mm to 1.32 mm with a mean value of 0.94 $\pm$ 0.18 mm. Sepal length mean value was 4.83 $\pm$ 1.85 mm; a variation of 2.74 mm to 10.10 mm was observed; average sepal width was 1.35 $\pm$ 0.45 mm; it varies from 0.75 mm to 2.21 mm. Days to flowering mean value ranges from 33 days to 157 days (Table 2). Kruskal-Wallis test was performed for all floral traits observed for all accessions of Horsegram. Traits such as pollen diameter showed significant variability (χ^2 = 45.75, $p < 0.001$, df = 24) across horsegram accessions collected from different countries (Table 2).

3.3. Ovule and pollen number, size and morphology

The flower is hermaphroditic with the following flower formula: 5S + 5P + (9 + 1) E + 1C. In accessions from Benin we found in each carpel five ovules of the same shapes and size (Fig. 3A). In contrast, Horsegram 250E collected from Nepal (Asia) showed a different number of ovules per ovary. In each carpel, there were six ovules of different sizes (Fig. 3B). Accessions from Australia and Indian (Liechardt1–3 and CPI22679) showed a different number of ovules. In each carpel, there were seven ova of different sizes (Fig. 3C).

Pollen types also varied according to the origin. Type A pollen was found in accessions from Benin and were highly similar except in size. Type A is sub-spheroidal or circular, triangular in polar and elliptical in equatorial view. The pollens are tricolporate (3 complex aperture) in nature. The exine has a reticulate structure and the tectum is rugulate (or scabrous) and thick without spines (Fig. 4A). The average diameter of type A pollen is 39.8 μm ~ 40 μm. Horsegram 250E also showed pollens which are sub-spheroidal or circular, subtriangular in polar view and elliptical in equatorial view. However, this accession possesses the type B pollen which consists of triporate pores, an echinulate structure with tuberous spines (Fig. 4B). The tectum is ornamental because of the presence of spines (pollens are supratectate). The average diameter of the pollen is 55.5 μm. Accessions from Australia and Indian (Liechardt1–3 and CPI22679) also exhibit sub-spheroidal or circular pollens, subtriangular in polar and elliptical in equatorial view. The pollens are found to be type B (Fig. 4b), triporate in nature and the exines are echinulate with spines.

3.4. Pollen viability and stigma receptivity

Pollen viability was considered based on the pollen type observed. During the day (7 h, 11 h and 15 h), the viability of accessions with type A pollen is significantly high (Fig. 5) but reduced at 7 pm (Fig. 5).

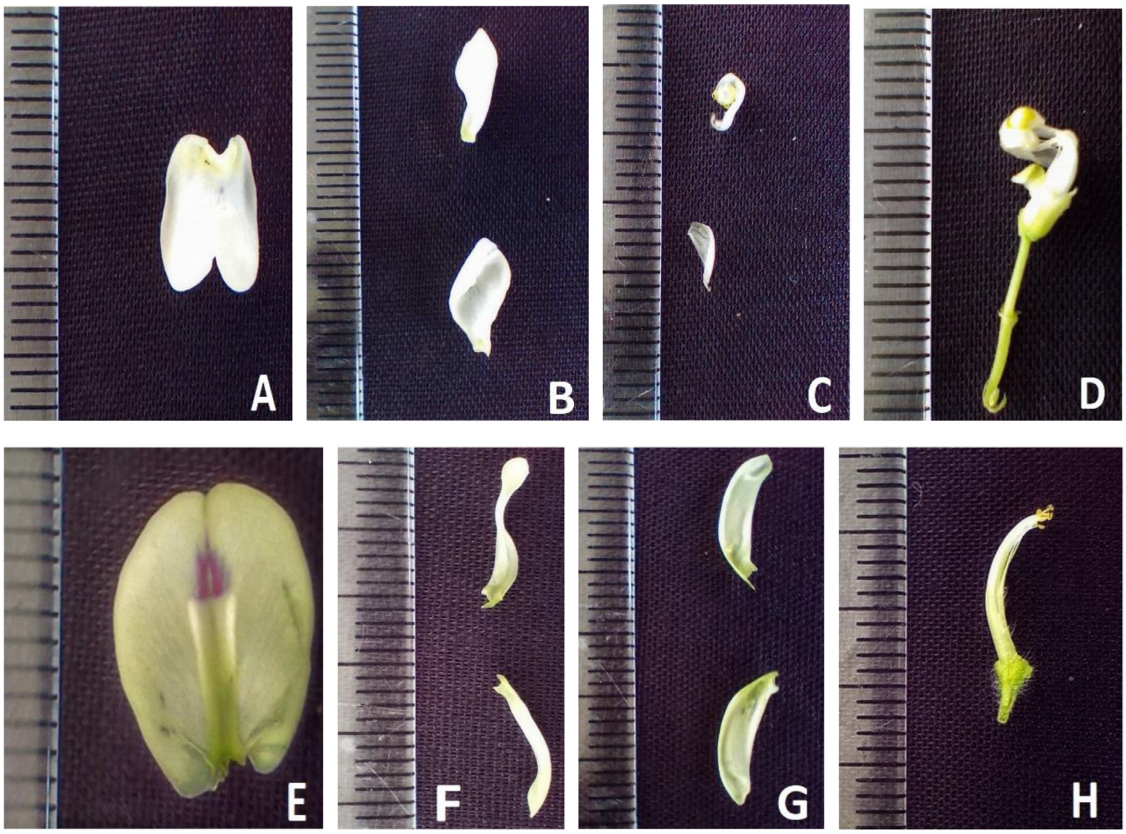

Fig. 2. Variation in different organs of horsegram flower collected from Benin, Mali, Tanzania, Australia, Nepal, and India. A-E-Standard, B-F-Wings, C-G-Keels and D-H-Gynoecium and Androecium. Organs A to D are found in accessions from Benin. E to H are found in accessions from other countries. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Descriptive statistics and Kruskal-Wallis test for floral traits in horsegram (*Macrotyloma uniflorum*).

	Pollen diameter (μm)	Stamen length (mm)	Pistil length (mm)	Ovary length (mm)	Ovary width (mm)	Sepal length (mm)	Sepal width (mm)	Standard length (mm)	Standard width (mm)	Peduncle length (mm)	Bud Length (mm)	Days to flowering (days)
(Mean ± SD	43.76 ± 8.65	8.07 ± 0.83	11.74 ± 0.89	5.80 ± 1.17	0.94 ± 0.18	4.83 ± 1.85	1.35 ± 0.45	8.92 ± 2.46	7.82 ± 1.09	16.30 ± 7.55	7.50 ± 3.26	50.38 ± 30.05
Min	30.50	4.78	8.79	4.10	0.58	2.74	0.75	5.41	5.25	6.01	4.64	33.00
Max	68.00	9.14	13.33	8.75	1.32	10.10	2.21	13.91	11.20	34.63	14.53	157.00
χ^2 (df = 24)	45.75	34.05	35.69	40.71	33.48	42.13	41.49	45.25	35.07	42.41	44.38	39.30
p-value	0.009	0.133	0.097	0.033	0.148	0.024	0.027	0.011	0.110	0.022	0.014	0.045
CV (%)	19.77	10.29	7.58	20.17	19.15	38.30	33.33	27.58	13.94	46.32	43.47	59.65

Degree of freedom: 24, χ^2 = Chi-square value, CV: Coefficient of variation%, SD: Standard deviation, Min: Minimum value, Max: Maximum value.

A similar situation was experienced in accessions of type B pollen. In this case, pollen viability reduced drastically as compared with other time ranges. A high percentage of pollen viability was observed during the daytime; however, at 7 pm the viability is slightly lower for both pollen types (Fig. 5). The in-vitro germination of pollen was also observed and estimated based on the pollen types. Type A and Type B showed close performance in the germination when subjected to 10% sucrose solution as substrate. The germinability of the pollen were further examined by accessing the percentage growth of pollen tubes from the pollens of both type A and B. It was observed that the pollen types showed similar pollen tube growth rate. This indicated the uniform pollen growth in Horsegram.

Introduction of 3% hydrogen peroxidase on stigma and other components of gynoecium found in accessions with Type A and Type B pollen revealed the presence of bubbles during dehiscence of bud (Fig. 6A) and immediately the flower entered anthesis phase (Fig. 6B).

3.5. Variation and correlations among quantitative traits of reproductive organs

Pollen diameter in the accessions from Australia was wider than other accessions from another geographical region (Fig. 7b). Accessions having type A pollen mature early than others ($p < 0.001$). Pistil length was lengthier for Australia accessions compared to other accessions from other eco-geographical regions (Fig. 7d). Accessions from India and Tanzania had flower with longer ovary ($p < 0.001$), whereas flower of accessions from Republic of Benin had the shortest ovary (Fig. 7e).

Analysis of floral traits such as sepal length and width showed that accessions from India had longer and wider sepal as compare to other accessions from other country. Accessions from the Republic of Benin exhibited longer peduncle ($p < 0.001$) as showed in (Fig. 7i). Accessions from India had longer bud ($p < 0.001$), followed by accession from Nepal; small bud was observed in accession from the

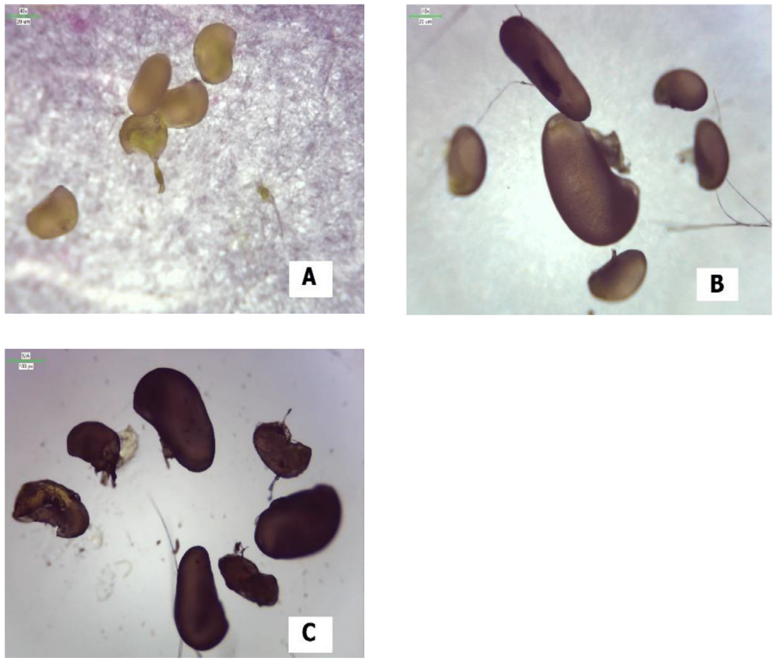

Fig. 3. Variation in the number of ovules per ovary in horsegram. **A**- Number of ovules in accessions from the Republic of Benin; **B**-Number of ovules in accessions from Nepal and **C**-ovules found in accessions from Australia, Mali and Tanzania. Jeulin® □ light microscope with integrated camera at X40O and X1000 magnifications. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

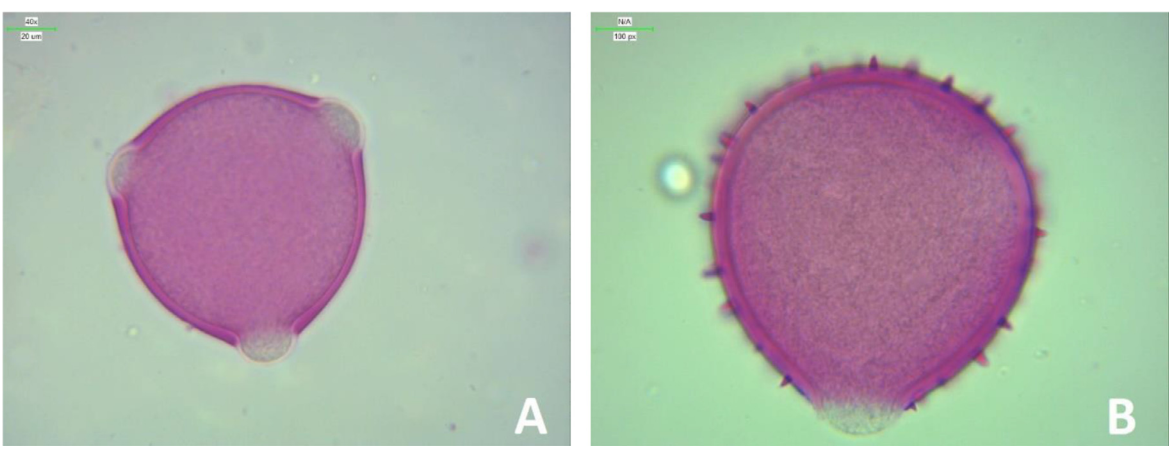

Fig. 4. Variation in pollen structure in horsegram accessions. **A** : Type A (Pollen structure found in accessions from Republic of Benin). **B**: Type B (Pollen structure found in accessions from Nepal, India, Australia, Tanzania, and Mali). Jeulin® □ light microscope with integrated camera at X40O and X1000 magnifications. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Republic of Benin (Fig. 7j). Smaller standard length was observed in accessions from Benin ($p < 0.001$).

Significant positive correlations were between sepal length and ovary length ($r = 0.84$), pollen diameter ($r = 0.78$), days to maturity ($r = 0.64$) and the maturity traits; days to flowering ($r = 0.69$). Correlation between pollen diameter and all other traits were highly significant except for stamen length, pistil length, ovule width and sepal width (Fig. 8). Standard length exhibited highly significant correlation with sepal length ($r = 0.91$), ovary length ($r = 0.86$), pollen diameter ($r = 0.83$), days to 50% flowering ($r = 0.64$).

4. Discussion

4.1. Floral behavior

Horsegram exhibits indeterminate flowers. Floral and maturity traits are variable among different group of accessions. The study also revealed the different phases ranging from young primordial bud initiation to the anthesis phase. Knowledge of floral biology has been a very crucial step to achieving a successful hybridization in legumes and particularly in horsegram. As reported by Bhave and Dhonukshe (1991), the use of hybridization is very limited in horsegram and the challenges were attributed to the small sizes of its flowers. Bhave and Dhonukshe (1991) dwelled into variability study of floral attributes, then suggested a proper study of the floral attributes such as the pollen biology and the stigma receptiveness. Detailed information regarding pollen morphology and behavior, stigma receptivity and in-vitro pollen germination of horsegram was provided to in this study.

Successful pollination and accomplishment of higher yield depends on the quality of pollen (Gupta et al., 2017). We found that the architecture of the estivation of petal in horsegram accessions collected from Benin could make the emasculation and pollination process more difficult than in accessions from other regions. The

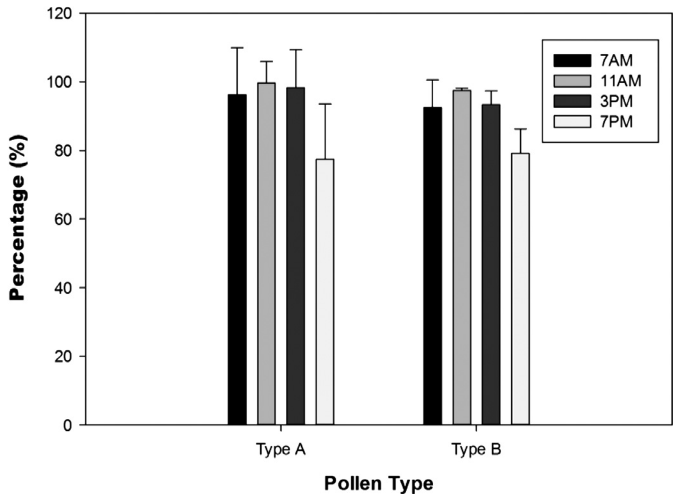

Fig. 5. Behaviour of pollen viability across the two types of pollen observed. Type **A**- Accessions with the non-echinates pollen and Type **B**- Accessions with echinates pollen.

whorl arrangement of the keels with the androecium and gynoecium makes it difficult to separate them; these could lead to the breakage of the structure and eventually lead to the loss of the flower. These findings suggest that accessions from the Republic of Benin should be incorporated into the breeding program as the male parent line and other accessions or cultivars from other ecological regions should be emasculated. However, there is a high difference between the Benin accessions and the other ones suggesting that there might be hybridization barriers between the two types.

Floral and maturity traits exhibit a high degree of positive correlation which could be used in the diversity study of this species. A highly positive correlation between pollen diameter and days to flowering denotes the importance of pollen diameter in the selection of early flowering accessions of horsegram. This implies an increase in the yield harvest of the early flowering accessions since the plant flowers all through the development phases.

4.2. Pollen ultra-structure, viability and stigma receptivity

Pollen viability is a major prerequisite to successful pollination and seed set in angiosperm (He et al., 2017). This study depicted the pollen viability at different time range and state the receptivity of stigma in horsegram accessions. Pollen viability result showed that accessions with type A and type B pollens revealed highly viable pollens from 7 h am till 3 pm but reduced viability as they approached night around 7 h pm. This showed that breeders should concentrate on the pollination process during the day. Further study will focus on considering fewer interval hours and extension around the hours of a day during anthesis to understand the behavior of horsegram pollen throughout those hours of the day.

We found that most accessions of horsegram evaluated for pollen viability performed more than 70% during the day but showed moderate in-vitro germination rate during anthesis. This study also authenticates the importance of the 2% acetocarmine stains and the 10% sucrose solution as a highly discriminating reagent for testing for pollen viability and in-vitro germination respectively. Although, other diverse discriminating reagents have been adopted in recent reports and included Tetrazolium stain, Aniline blue, Alexandra stain and Iodine potassium iodide test (I^2KI). Recently, 15% sucrose + 0.25% Boric acid + 300 mg calcium nitrate was authenticated by Rathod et al. (2018) as the best medium for pollen germination in *Momordica* spp. He et al. (2017) also suggested the use of controlled pollination but Kumar et al. (2020) identified acetocarmine to be effective for wild marigold. In accordance with the recent report of Kumar et al. (2020), acetocarmine exhibited a high degree of discriminating power and this result will significantly promote breeding of horsegram through hybridization.

The study revealed pollen structure as one of the discriminating factors for horsegram. The variability in the pollen structure suggests two taxa within the crop. The first pollen type is found in accessions collected from Mali, Tanzania, Australia, Nepal, and India. It was referred to as type B pollen with echinate attribute; that trait can be used as a marker to characterize horsegram from other regions of the world. Pinheiro et al. (2018) emphasized on the importance of echinate pollen as a defense mechanism that evolved for preventing high loss of pollen during the activities of pollinators. Also, Konzmann et al. (2019) stated that some species of Fabaceae has developed protective mechanisms which includes pollen and transformed petal. As reported by Balan et al. (2016) who regarded pollen grain as a biological entity that deserves special attention in making taxonomic decisions because pollens are subject to less environmental variations. Pollen morphology have hugely contributed in solving the mystery of phylogenetic lineage in groups like Rosaceae based on traits such as pollen shape, aperture structure, aperture number and exine sculpturing pattern (Elisens and Skvarla, 2010). Those traits are known to be important lineage markers among genera (Elisens and Skvarla,

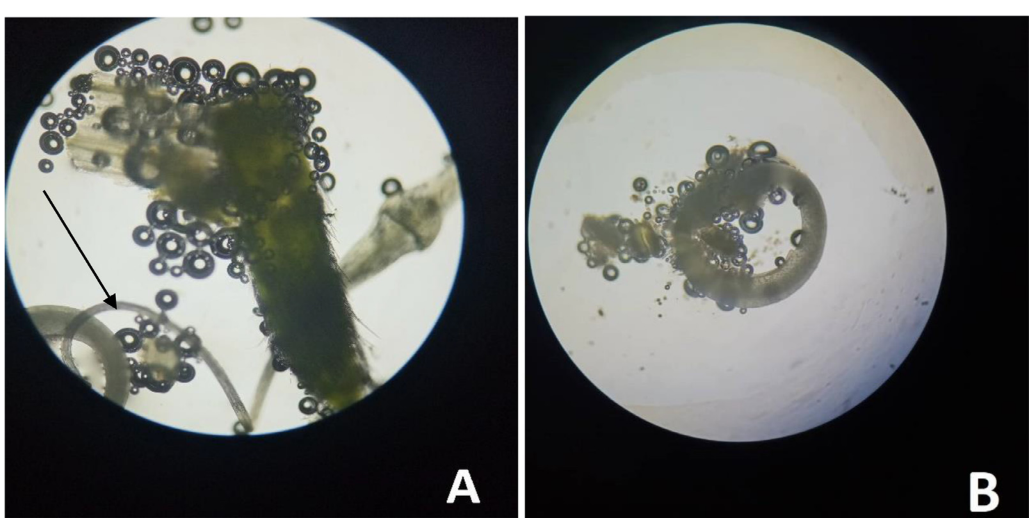

Fig. 6. A. Stigma receptivity during budding and anthesis phases. B. Stigma receptive image at dehiscence bud phase (Arrow to stigma). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

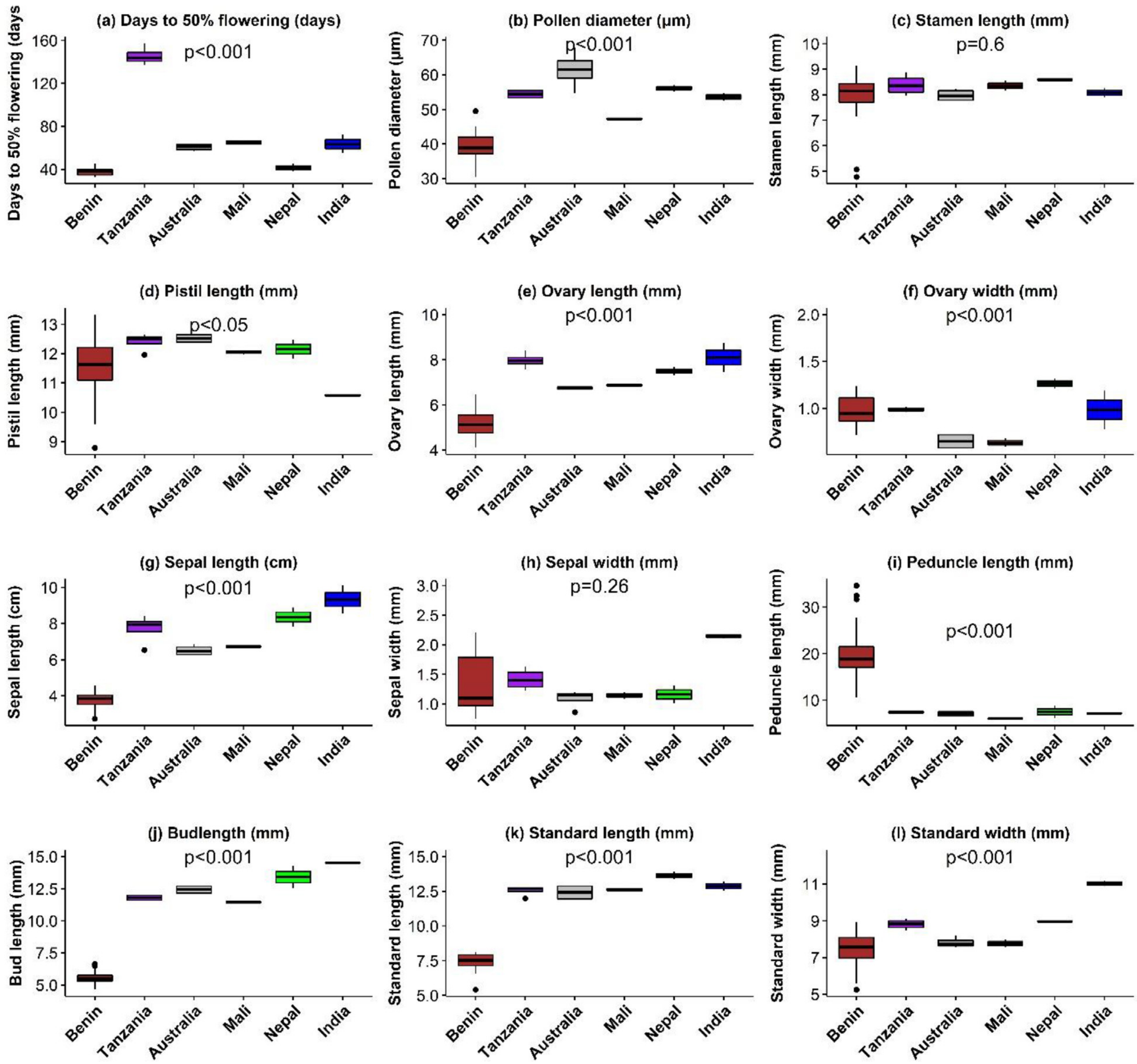

Fig. 7. Variation of traits in Horsegram based on country of origin. (a) Days to 50% flowering; (b) pollen diameter; (c) stamen length; (d) pistil length, (e) ovary length; (f) ovary width; (g) sepal length; (h) sepal width; (i) peduncle length; (j) bud length; (k) standard length; (i) standard with. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

2010). We then suggest the use of pollen structure in the taxonomic study of diverse collection of horsegram for differentiation.

Activities of enzymes such as 3% hydrogen peroxidase are effective in indicating the receptiveness of the stigma which is crucial in an hybridization program (He et al., 2017). Information on the stigma receptivity of horsegram was still unknown and this study is the first-ever to reveal that knowledge. High stigma receptivity before (budding at dehiscence phase) and during anthesis is a sign of proper ability and readiness of the stigma to accommodate the pollen for a successful pollination process. We found that stigma was receptive at the bud dehiscence phase and during anthesis. This clue denotes the access to pollinate immediately the flower opens (at anthesis). Furthermore, diversity in the number of ovules per ovary can also be used as an important selection criterion in selecting parents during the breeding program. Bhave and Dhonukshe (1991) underlined the variation in the number of ovules per plant but none of the horsegram accessions used in his study had more than five ovules per ovary. That fact is in contrast with the findings in this study where six and seven ova per ovary were observed. This crucial information revealed accessions that produce more ovules which is equivalent to the number of seed to be produced. Incorporating accessions sourced from Australia (Leichardt 1 and 2) and India (CPI22697) which have seven ova will eventually lead to the improvement of horsegram yield.

5. Conclusion

In this study, we generated new knowledge about the different stages of floral development, the structure of the androecium and gynoecium, the morphology of the pollen, and the right time for emasculation and pollination. The study clarified the discrepancies in floral traits observed among accessions from various origins. This knowledge will rightly help breeders build up a program for cultivar development.

The accessions of horsegram used in this study were collected from six countries namely Australia, Nepal, India, Tanzania, Mali, and Benin. The hypothesis of the existence of significant variations among those accessions in terms of floral structure and reproductive

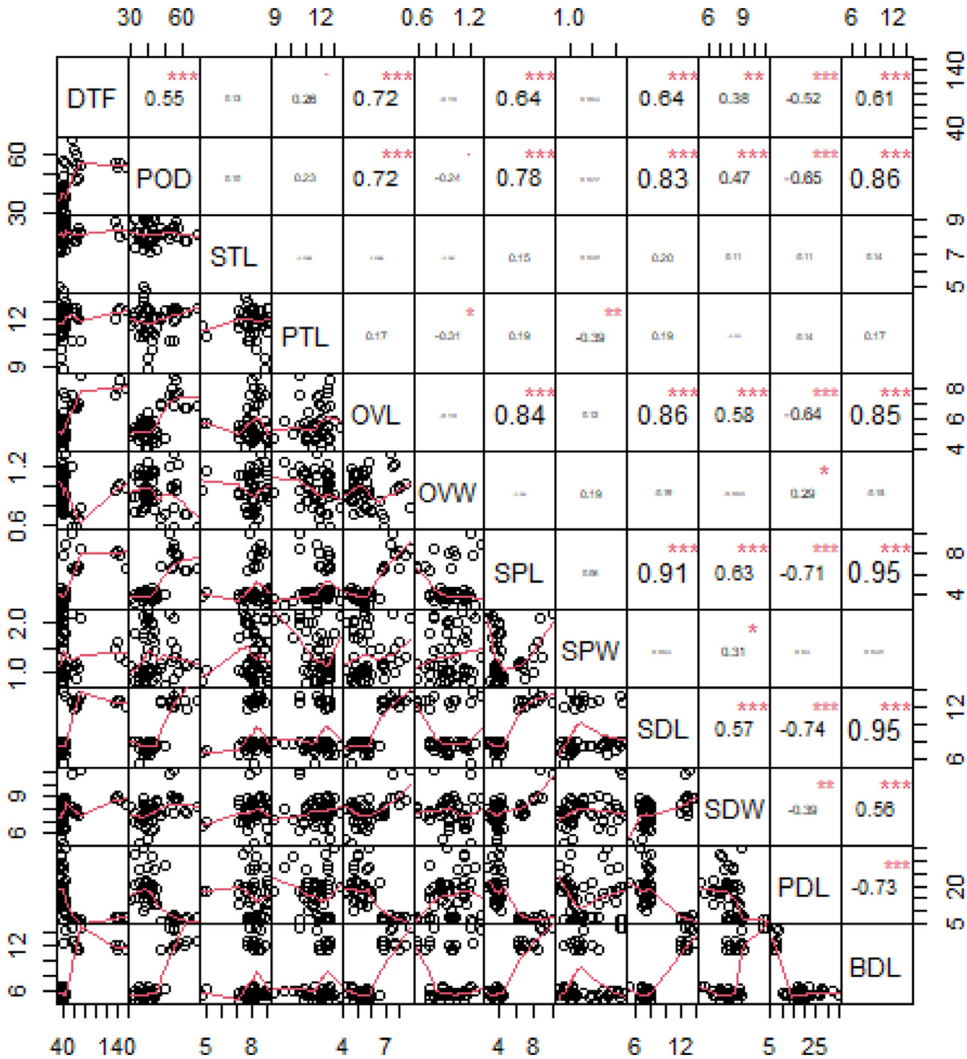

Fig. 8. Correlation matrix among floral quantitative traits. DTF = Days To Flowering, POD = Pollen Diameter, STL = Stamen Length, PTL = Pistil Length, OVL = Ovary Length, OVW = Ovary Width, SPL = Sepal Length, SPW = Sepal Width, SDL = Standard Length, SDW = Standard Width, PDL = Peduncle Length, BDL = Bud Length. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

architecture held true. Those accessions exhibited two major floral and pollen types. For instance, all accessions from Benin showed white standard, white petal and carpel with five ovules of similar shape and size. The pollen of Benin accessions are sub-spheroidal or circular, triangular in polar and elliptical in equatorial view and tricolporate in nature. Pollen diameter is 39.8 μm ~ 40 μm. Accessions from other countries exhibited the second type of floral and reproductive architecture. They have a yellow standard with a purple dot, green-yellow petals, and carpel with six to seven ovules with different sizes. The pollen consists of tri-porate pores, an echinulate structure with tuberous spines. The average diameter of the pollen is 55.5 μm.

In general, pollens were highly viable from 7 h am till 3 pm but reduced viability was observed at night time around 7 h pm. Stigma receptiveness occur not only at anthesis stage; this contradicts our second hypothesis. Stigma was receptive at the bud dehiscence phase and during anthesis. Crosses between accessions from Benin and accessions from other regions would result in promising hybrids. However, two taxa are suspected in the plant materials studied.

Authors' contributions

IAI, COAA and EGAD: Conceptualization. IAI and COAA: Investigation, Methodology, Data curation, Formal analysis. CAOA: Investigation, Methodology. IAI: Writing – Original draft; JOA, CAOA, NFH and EGAD: Writing – review and editing. EAD: Funding acquisition, Supervision; Validation; Project administration.

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Consent for publication

Not applicable.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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