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Research article

Understanding floral biology and pollination behavior of *Stylosanthes* spp. to enhance improvement programs

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Abstract

The significance of a range legume *Stylosanthes* spp. is long established in grassland ecosystems. However, its potential for the nutritional security of livestock remains underexploited. A better understanding of floral behavior will assist in its improvement through improved hybridization techniques, seed production and breeding schemes. Only limited studies have been conducted on the floral biology of *Stylosanthes* spp. The current study focused on floral morphology, anthesis, longevity and pollen viability of four *Stylosanthes* spp. *viz., S. hamata, S. seabrana, S. viscosa* and *S. scabra*. The anthesis time varied among the *Stylosanthes* species studied here and the maximum anthesis occurred during Indian Standard Time (IST) from 8.00 AM to 10.00 AM. The flower longevity of *Stylosanthes* spp., lasted for a day after the anthesis. All the species were found to be protandrous, as pollen dehiscence occurred 1 to 2 hours before anthesis. The time of the highest pollen viability (85.08 ± 2.16%) coincided with stigma receptivity. After two hours of anthesis, higher receptivity and maximum activity of stigma was observed in *S. hamata* compared to other *Stylosanthes* species. Furthermore, *S. scabra* showed the maximum pollen count while estimating a number of pollen per anther and flower.

Keywords: Pollen viability, Range legumes, Stigma receptivity, *Stylosanthes*

Introduction

Range legumes are important agricultural resources supporting livestock production systems in the world's arid and semi-arid regions. Besides its role as pasture in semi-arid tropics, it provides ecosystem services like global warming mitigation, soil conservation and improvement (Singh *et al*., 2018). *Stylosanthes* (Stylo) is one of the important perennial legumes native to central and South America, majorly grown in tropical regions. Due to its soil fertility restoration capacity, it plays a vital role in developing wastelands in India. Due to its capacity to remain green under dry conditions, stylo is particularly suited for forage in sub-humid tropical and subtropical areas (Kumbhar *et al*., 2009). It can be used as feed for all types of animals in the form of green fodder, hay and silage, as it has excellent nutritional value and palatability. It has 12 to 18% crude protein (CP), 47 to 55% neutral detergent fiber (NDF) and 34 to 40% acid detergent fiber (ADF) contents. *Stylosanthes* also serve as an affordable and sustainable source of important minerals, *viz.* calcium (10–15 g/kg DM), phosphorous (2–2.8 g/kg DM), zinc (15–20 mg/kg DM) and copper (8–10 mg/kg DM) (Kumar *et al*., 2016).

Nearly all species of the genus *Stylosanthes* are mainly diploid (2n = 20) except *S. scabra,* an allotetraploid species. (2n = 40) (Coates *et al*., 1997). *S. hamata, S. seabrana, S. viscosa* and *S. scabra* are considered as important species (Marques *et al*., 2018), which are used as a range legumes in India. The inflorescence of *Stylosanthes* is a cluster of yellow papilionaceous flowers with a terminal portion consisting of 2 to 3 alternate spikes (Kumar *et al*., 2016). The indeterminate growth habit with non-synchronous flowering and seed maturity causes seed shattering, thus leading to significant seed losses. Besides, constraints like poor germination and lesser seed yield under arid conditions discourage *Stylosanthes* cultivation (Chandra *et al*., 2006).

Investigations on flower morphology, phenology, longevity and pollen viability facilitate an improved understanding of the reproductive behavior of the crop,

which can be used to accelerate the progress of genetic improvement of *Stylosanthes* spp. As there is a wide gap between the demand and supply of forage seed in the country, the floral biology information may be helpful for various stakeholders to bridge the gap in the supply chain (Kumar *et al*., 2021). Recent advancements in floral biology of *Stylosanthes guianensis* have brought new insights, particularly focusing on genetic mechanisms governing flowering under stress and identifying the FT gene (Wang *et al*., 2024). The study highlighted *S. guianensis* flowering patterns and how certain transcription factors (*e.g.,* SgRVE1) are involved in regulating floral initiation and cold stress responses (Wang *et al*., 2024). *Stylosanthes* is also valued for its role in improving soil health and providing forage. Various studies were conducted to increase seed yield and to improve the genetic diversity, which are crucial for sustainable agriculture, especially in tropical and subtropical regions. However, limited attention has been given to understand the flowering phenology of the genus *Stylosanthes.* Insights from studies on floral biology contributed significantly to optimizing improvement strategies based on the reproductive behavior of flowering plants. In this context, pollen viability and stigma receptivity are crucial for a successful seed set (Fu *et al*., 2017). Pollen germination tests or pollen staining techniques can assess pollen viability. Pollen staining techniques are preferred over other methods due to their ease of use and quick results. However, they sometimes appear to overestimate the viability because they often stain dead pollen grains (Abdelgadir *et al*., 2012; Gaaliche *et al*., 2013). Therefore, ascertaining the suitability of the pollen staining method for *Stylosanthes* spp. is of great significance.

The longevity of anthesis and floral senescence are also critical to the reproductive success of plant spp. (Aximoff and Freitas, 2010). Understanding flower longevity would facilitate a resource allocation strategy. Increasing flower longevity could contribute to fulfilling the reproductive success of species (Ashman and Scheon, 1994). Our current research examined variations in flower timing and pollination behavior of four species of *Stylosanthes* New knowledge about floral biology and its influence on the reproductive behavior of *S.* species will help to guide the crossing program and hybridization decisions in *Stylosanthes*.

Materials and Methods

***Species and study area*:** Four *Stylosanthes* spp. *viz*., *S. hamata, S. seabrana, S. viscosa* and *S. scabra* were established in the Technology Demonstration block (Each plot size 31m x 7.5 m) at ICAR-IGFRI, Jhansi (25.511° N latitude, 78.534° E longitude and 271m msl). The mean annual precipitation at the study site ranges from 680 to 900 mm with a mean annual temperature of 31.7°C. This study was performed from September to December 2018. The samples were collected from all the species and directly used for further studies.

***Morphometric features of flowers*:** The floral parts from fully opened fresh flowers of different *Stylosanthes* spp. were measured (n =10) and photographed using a stereo binocular microscope (Leica M2500LED with automountage Leica MC170HD camera). The number of pollen per anther and flowers was estimated by dilution technique (Dieringer, 1994).

***Flower longevity*:** About 30 flower buds were tagged and monitored daily from September to December 2018 to study flower longevity. To examine the flower opening pattern and record the time of anthesis, the flower buds that were going to open the next day were observed at hourly intervals (IST 6:00 AM to IST 6:00 PM). The duration between flower opening to shriveling was recorded and the optimum longevity of each flower species was worked out (Vesprini and Pacini, 2005). Anthers from 10 tagged flowers were collected at different time intervals on the day the flower opening was examined to study another dehiscence pattern.

***Pollen viability*:** A preliminary study was performed to find a suitable staining method for estimating the pollen viability of four *Stylosanthes* spp. The fresh pollen and heat-killed pollen (2 h at 80°C) were taken, and different staining techniques *viz*., Baker (Dafni, 1992), MTT (Marks, 1954), TTC (Norton, 1966) and IKI (Baker and Baker, 1979) were evaluated to find out the suitable method which unambiguously differentiates between the dead and the viable pollen in *Stylosanthes*. After identifying the appropriate staining technique (Baker's method), pollen viability of *Stylosanthes* spp. was ascertained at different time intervals, starting from anthesis to shriveling of flowers. The viable and non-viable pollen grains were identified based on the staining pattern and the pollen viability percentage was calculated based on a total number of view fields in the microscope (Leica DM2500 LED).

Stigma receptivity: The stigma receptivity of selected Stylosanthes spp. was assessed using the peroxidase test as described by Dafni and Maues (1998) with slight modification. To determine the stigma receptivity, the flowers at three different stages, *viz*,. two days before anthesis, one day before anthesis and on the day of anthesis (at five different times up to shriveling of flowers), were randomly selected and brought to the laboratory. The stigma from the fresh flower was carefully removed and observed under a magnifier (´30) for the presence of pollen and for any damage to the surface, either of which may cause enzymatic activity regardless of stigma receptivity (Dafni, 1992; Kearns and Inouye, 1993). The pistils were then placed on a glass slide

containing a fresh H_2O_2 solution (1%) drop and viewed under a Leica DM2000 (HI PLAN 100X/1.25 DIL). The presence of oxygen bubbles on the stigma was observed. The bubbling from the stigma was considered a mark of receptivity and more bubbles denoted stronger stigma receptivity.

***Statistical analysis*:** Statistical analysis was performed for all morphometric features by using SPSS software, and measured variables were presented as mean ± SD. The mean value of flower opening patterns, stained pollen percentage and pollen viability % were analyzed by using MS Excel 2010.

Results and Discussion

***Floral traits*:** The reproductive biology of *S. hamata, S. seabrana, S. viscosa* and *S. scabra* was analyzed in reference to flower morphology, anthesis, stigma receptivity and pollen viability. In the present study, we documented critical floral traits essential for pollination and helped to differentiate among the different species of *Stylosanthes* (Ornduff, 1969). *Stylosanthes* is a monoecious plant with odorless and bright yellow flowers. As a Fabaceae member, its flower has a typical cleistogamous structure composed of five fused sepals and five free, yellow-colored petals (standard, wings and keel) with zygomorphic symmetry (Kumar *et al*., 2016). The gynoecium consists of one stigma, style and ovary, whereas the androecium consists of 10 anthers (5 long + 5 short) and filaments (Fig 1A-F). All *Stylosanthes* spp. consists of 10 stamens. Interestingly, the development of five long anthers was completed 1-2 days earlier than the remaining five short anthers. The staggered maturity of pollen grains enables the continuous supply of viable pollen to stigma to ensure self-pollination. Among many factors, flower fragrance was also associated with pollination (Petren *et al*., 2021). Since all the studied *Stylosanthes* species had odorless flowers, our findings strongly concorded with Raguso's (2016) observation that the floral scent is not an essential criterion for self-pollination.

Besides, a few more important flower morphological traits were also recorded from a pollination perspective. The length of a fully opened flower was higher in *S. scabra* (6311.27 µm) followed by *S. hamata* (6190.47 µm), while the minimum length was recorded in *S. viscosa* (6004.47 µm) (Table 1). The bud length and width of the flower during pre-anthesis varied between 3505.41, 3638.98, 958.72, and 1014.16 µm, respectively. The highest style length (2167.64 µm) and width (119.75 µm) were recorded in *S. scabra*. The highest and lowest anther filament lengths were recorded in *S. scabra* (1381.38 µm) and *S. seabrana* (1290.65 µm) spp., respectively (Table 1). *S. hamata* had the highest anther lobe length (521.66 µm) and width (280.78 µm). The pollen grains were abundant in *S. scabra* (265.15 and 3034.73 per anther and flower, respectively) compared to other *Stylosanthes* spp. (Table 1). Examination of floral morphological features suggested a clear difference among different *Stylosanthes* spp. Our findings, like bigger flowers (in *S. scabra*) had higher pollen grains than small-sized flowers, strongly agreed with the earlier research (Galen, 1999) demonstrating a positive correlation between flower size and pollen amount. The matured seed length and width were higher in *S. seabrana* (4803.88 and 1909.09 µm), whereas the lowest was observed in *S. scabra* (4426.34 and 1869.07 µm, respectively). In *Stylosanthes* species, floral traits such as flowering period, flower longevity, color, odor, anthesis time, size of floral organs, pollen production, pollen viability and stigma receptivity played critical roles in pollination and seed production. More extended flowering periods increased the chance of repeated pollinator visits, leading to a higher seed set. However, asynchronous flowering within populations might reduce cross-pollination and lead to more self-pollination, affecting genetic diversity and seed vigor (Fenster *et al*., 2004). Longer flower longevity extends the window of opportunity for pollen deposition and fertilization (Ashman and Schoen, 1997). It plays a major role in environments with fewer pollinators or irregular pollinator visits, as seen in *Stylosanthes*. In *Stylosanthes*, bright or contrasting colors (yellow) can attract generalist pollinators and UV patterns on petals

Fig 1. (A and B) Bud stage of flower; (C) Mature flower bud before anthesis; (D) Fully opened flower; (E) Shriveled flower; (F) Immature seed; (G) Matured hooked-shaped seed

(invisible to humans but visible to pollinators like bees) could guide pollinators to the reproductive organs of flowers (Chittka and Menzel, 1992). Dudareva *et al*. (2004) highlighted the role of floral scents in mediating plant-pollinator interactions, showing how specific compounds attract particular pollinator species. Gomez and Zamora (2006) discussed the correlation between flower size and pollinator visitation rates, showing that larger flowers often attract more frequent and diverse pollinators. Similarly, Kalisz *et al*. (2012) reported that floral organ morphology influenced self-pollination and outcrossing rates in flowering plants.

***Flower onset and longevity*:** Flowering in *Stylosanthes* occurs between September and December in India. A minor blooming period is also observed during the onset of summer in the months of April and May in all species of *Stylosanthes* studied. In this experiment, an attempt was made to study the sequence of flowering events in each species of *Stylosanthes*. Synchrony in the onset of flowering was observed for all four species. Anthesis in *Stylosanthes* species was observed between IST 6.00 AM and IST 11.00 AM, with a peak activity recorded between IST 8.00 AM and IST 10.00 AM (Fig 2).

The *S. seabrana* showed late anthesis around IST 10.00 AM among all species. Furthermore, it was observed that anther dehiscence occurred 1 to 2 hours before the anthesis in each species, and the dehisced pollens were found viable, which confirmed the protandrous nature of *Stylosanthes*. The flower buds matured in 2 to 3 days, and the flowers shriveled and withered on the day of anthesis after pollination (Fig 3A-E). Based on individual flower lifespan, the flower longevity was calculated for all four *Stylosanthes* species. The length of a blooming period in *S. hamata*, *S. scabra*, *S. seabrana* and *S. viscosa* was 9.56, 10.21, 9.63 and 9.29 hours, respectively (Table 1). After the completion of pollination, it took 16 to 17 days to develop into a mature seed. Therefore, it could be said that flower longevity in these *Stylosanthes* species lasts up to less than one day. The flowers that open during early morning or evening hours when pollinators are most active can maximize the chances of pollination. Anthesis timing also ensures that flowers remain receptive when the likelihood of encountering pollinators is high. Kumar *et al.* (2024) provided evidence on how anthesis time in many plant species is synchronized with pollinator activity to maximize reproductive success in forage crops.

Our findings reiterate the importance of anthesis in flower phenological events of plants, which could be used in planning for future research and improvement of *Stylosanthes* spp. Our study facilitates an understanding of how ecological factors influence variation in flowering and its relevance in pollination (Lima, 2022). Examination of the duration of pollen exposure and stigma receptivity in *Stylosanthes* species led to the conclusion that both the receptivity and flower had shorter life spans. It's

Table 1. Summary of some morphometric features of different *Stylosanthes* species

Morphometric features	Mean ± SD			
	S. hamata	***S. scabra***	***S. seabrana***	***S. viscosa***
Flowering period	Sept-Dec/April-May	Sept-Dec/April-May	Sept-Dec/April-May	Sept-Dec/April-May
Flower longevity (hrs)	9.56	10.21	9.63	9.29
Flower color	Yellow	Yellow	Light yellow	Yellow
Flower odor	Not present	Not present	Not present	Not present
Anthesis time (max.)	8.00 AM	9.00 AM	10.00 AM	9.00 AM
Pre anthesis bud (L) (μm)	3589.2 ± 156.62	3638.9 ± 211.14	3505.4 ± 237.88	3601.2 ± 157.62
Pre anthesis bud (w) (μm)	963.2 ± 88.02	1014.1 ± 115.12	958.7 ± 73.61	970.4 ± 77.94
Flower (L) (μm)	6190.4 ± 244.56	6311.2 ± 353.04	6011.2 ± 334.16	6004.4 ± 522.17
Style length (μm)	2131.8 ± 121.34	2167.6 ± 144.46	1934.0 ± 253.25	2034.3 ± 183.07
Style width (μm)	119.5 ± 8.86	119.7 ± 7.42	117.2 ± 7.96	119.3 ± 4.43
Anther filament length (μm)	1336.4 ± 84.14	1381.3 ± 137.66	1290.6 ± 112.81	1313.9 ± 99.11
Anther lobe length (μm)	521.6 ± 48.90	513.8 ± 37.66	491.3 ± 55.52	516.9 ± 53.05
Anther lobe width (μm)	280.7 ± 19.15	268.5 ± 21.44	268.1 ± 15.17	271.8 ± 26.32
Matured seed (L) (μm)	4726.6 ± 621.73	4426.3 ± 615.75	4803.8 ± 701.87	4612.4 ± 673.83
Matured seed (w) (μm)	1894.9 ± 111.28	1869.0 ± 64.05	1900.0 ± 70.34	1898.8 ± 96.02
No. of pollens/anther	257.8 ± 46.77	265.1 ± 33.22	254.9 ± 59.69	236.9 ± 58.57
No. of pollens/flower	2656.9 ± 514.63	3034.7 ± 644.20	2883.2 ± 837.09	2233.5 ± 350.10

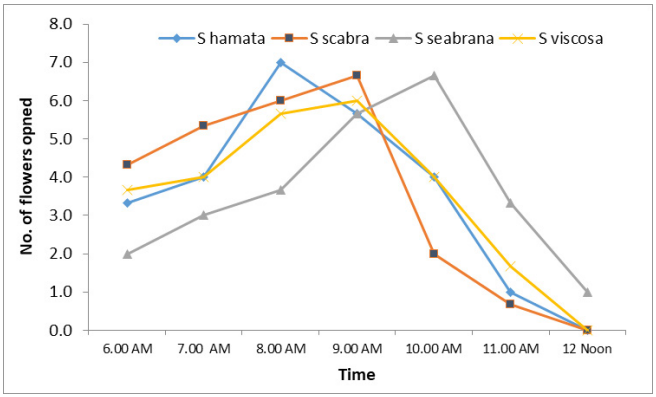

Fig 2. Flower opening (anthesis) pattern of four *Stylosanthes* species

a long-established fact that flower longevity is an important reproductive trait because longevity variation significantly influences fruit set, seed-ovule ratio and seed weight (Castro *et al*., 2008). The senescence of *Stylosanthes* flower was activated soon after the reception of pollen on the stigmatic surface. The pollination-induced early flower closure, reduction in nectar production and abscission of flower petals were also observed in many species (Van Doorn, 1997). Overall, a flower with an extended lifespan increased the opportunity for reproductive success but required high maintenance costs to sustain its functionality.

Flower longevity, while enhancing opportunities for pollination, demands significant resource investment from the plant, particularly in terms of water, energy and nutrients. Shorter-lived flowers often optimize reproductive success by efficiently timing pollen release and stigma receptivity to coincide with peak pollinator activity or favorable environmental conditions, as seen in species like *Stylosanthes* and other legumes. However, prolonged flower longevity could be advantageous in environments with unpredictable pollinator availability, though it might come at the cost of reduced overall seed production due to higher resource demands.

***Pollen viability*:** The study was also conducted to examine pollen viability and demonstrate differences in floral traits of different *Stylosanthes* spp. Pollen viability staining was used as a criterion to investigate the taxonomy of different genera (Zonneveld and Iren, 2001). A preliminary study was conducted using all pollen staining techniques, and the results showed that different methods stained both heat-killed and fresh pollen. However, staining of heat-killed pollen was varied, and the least viability was observed in Baker's method (9.58%) followed by MTT (16.54%), TTC (16.70) and IKI (23.37%). The highest pollen viability was recorded in TTC (82%) (Fig 4). *Stylosanthes* flowers with higher pollen counts increased the chances of successful cross-pollination. However, pollen viability must be high to ensure that pollen grains can successfully germinate on the stigma and result in fertilization.

Baker's method stained the lowest number of heat-killed pollen and also recorded the highest pollen viability (76.22%). Therefore, the study established Baker's method as the most appropriate for the estimation of pollen viability in *Stylosanthes* spp. In Baker's method, maximum pollen viability was observed at the time of anthesis (6.30 AM) in *S. viscosa* (85.08%) followed by *S. seabrana* (73.90%), *S. hamata* (67.05%) and *S. scabra* (55.24%) (Fig 5). At IST 2.30 PM on the day of anthesis, the viability declined to 27.70% in *S. viscosa*, 30.82% in *S. seabrana*, 9.17% in *S. scabra* and 5.10% in *S. hamata* (Fig 5). The longevity of *Stylosanthes* pollen was noticed up to 8 to 10 hours after anthesis. After that, it lost its viability because of shriveling and withering of flowers.

The four pollen staining methods, Baker, MTT, TTC, and IKI, could distinguish between fresh and dead pollen. TTC and MTC stained the highest number of fresh pollen as well as dead pollen. Abdelgadir *et al*. (2012) also reported that TTC method stained the highest percentage of fresh pollen in *Jatropa curcus*. Baker's method successfully differentiated dead and fresh pollen and stained the highest percentage of fresh

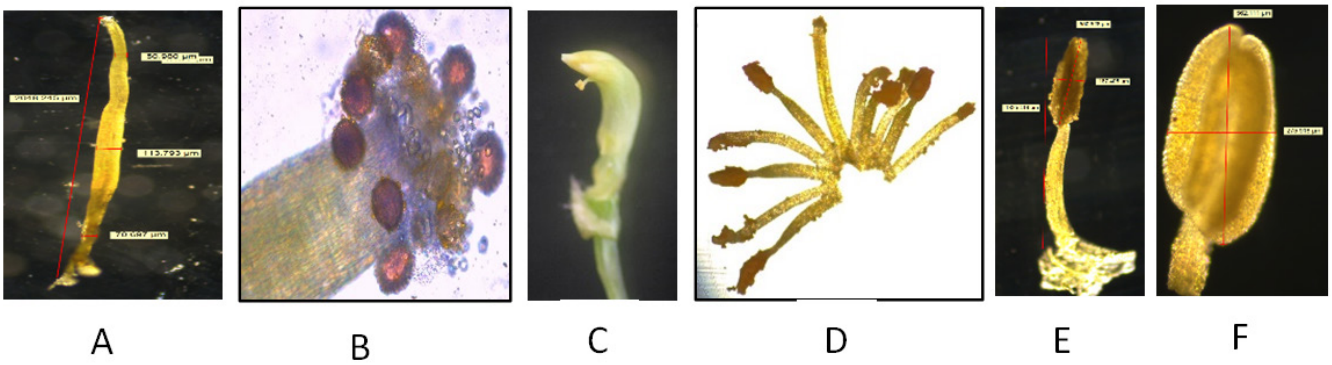

Fig 3. (A) Gynoecium part; (B) Stigmatic surface; (C) Stamen canal; (D) 5 long and 5 short stamens; (E) One stamens; (F) Anther

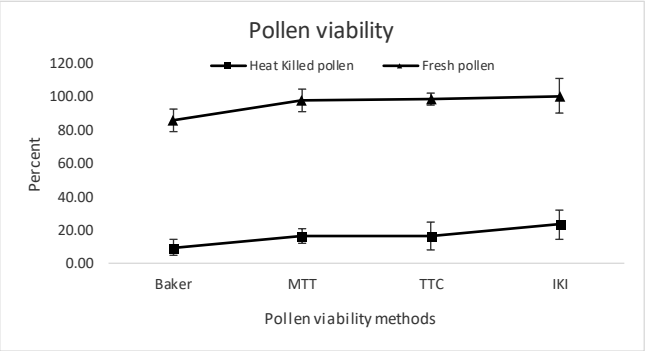

Fig 4. Stained pollen percent by different pollen viability methods

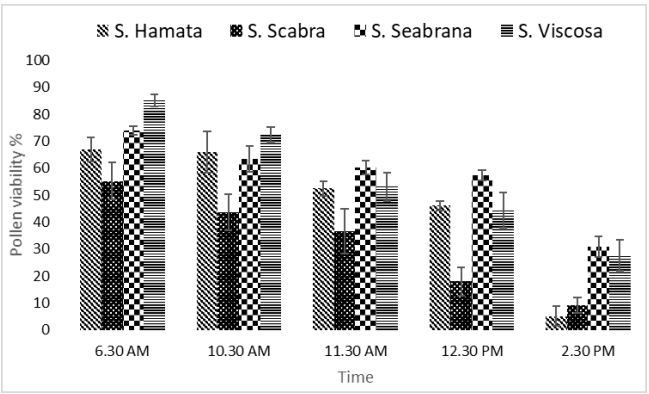

Fig 5. Pollen viability percentage of different *Stylosanthes* species

pollen and the lowest proportion of dead pollen. Thus, Baker's method could be used as a standard technique to test pollen viability in *Stylosanthes* spp. Baker's method has been established as the most suitable method to analyze pollen viability in other plant species, including *Cyclamen persicum* (Rodriguez and Dafni, 2000). Generally, *Stylosanthes* species show relatively brief periods of pollen viability, often ranging from a few hours to one or two days, depending on environmental conditions such as temperature and humidity. This is similar to other legumes and self-pollinating plants, where pollen viability could last a few hours to several days. In *Linum grandiflorum*, pollen viability typically lasts 24 to 48 hours under optimal conditions (Ghosh and Shivanna, 1980). Similarly, legumes like *Phaseolus vulgaris* (common bean) have a short window of pollen viability, around 1 to 2 days (Rose *et al*., 2023).

***Stigma receptivity*:** The receptivity of the stigma started from 8.00 AM on the 2nd day before anther dehiscence and maintained its receptivity till the 3rd day evening. The stigma receptivity was maximum between IST 8.00 AM and IST 10.00 AM in all the *Stylosanthes* spp. Thereafter, stigma receptivity showed a gradual decline (Table 2). In all species studied here, flowers started withering shortly after pollination, and the abscission of petals took place.

Pollen viability and stigma receptivity are prerequisites for successful pollination and seed set in any crop (Arathi, 2002). Besides, synchrony between pollen viability and stigma receptivity is essential for plant reproductive success. In all the *Stylosanthes* species, the maximum stigma receptivity was found on the day of anthesis (IST 8.00–10.00 AM). This observation was confirmed by a maximum number of bubbles produced in the peroxidase test, which coincided with the peak pollen viability in *Stylosanthes*. It confirmed the self-pollination behavior of all the *Stylosanthes* spp. Various research groups used peroxidase assay to test the stigma receptivity in Indian mustard, rice and wheat (Gupta *et al*., 2015). The present findings remained in line with the study of floral traits in relation to pollination biology. In *Stylosanthes* spp., pollen viability and stigma receptivity exhibited specific timing patterns that were critical to successful pollination and seed production, as in other legumes and self-pollinating plants. In flowering plants, a long period of stigma receptivity might increase the chance of successful fertilization, especially in variable pollinator environments. However, synchrony between pollen availability and stigma receptivity was crucial for maximizing seed sets. Harder and Barrett (1995) explored how variations in stigma receptivity influenced reproductive success in angiosperms, suggesting that longer receptivity windows often correlate with increased

Table 2. Stigma receptivity behavior of four *Stylosanthes* species

Stages		*S. hamata*	*S. scabra*	*S. seabrana*	*S. viscosa*	Total
2 Days before anthesis		+	+	+	+	+
1 Days before anthesis		+++	++	++	+++	+++
Day of anthesis	8:00 AM	++++	+++	+++	++	++++
	10:00 AM	++++	+++	++++	+++	++++
	12.00 Noon	+++	+++	++++	++	+++
	2.00 PM	++	++	++	++	++
	4.00 PM	++	+	++	++	++

*(+: No reaction; ++: Less reactivity; +++: Moderate reactivity, ++++: High reactivity)

seed production. Stigma receptivity in *Stylosanthes* is generally synchronized with the period of pollen viability, lasting around 2 to 3 days post-anthesis, which ensures successful pollination during this window. This timing was crucial because, like in many legumes, the overlap between pollen viability and stigma receptivity was what determined reproductive success. For instance, in species like *Grevillea robusta*, stigma receptivity peaks around two days post-anthesis, which is similar to *Stylosanthes*. The variations in flower color, such as those seen in other species, could significantly affect the type of pollinators that visit, with certain hues attracting different types of bees and butterflies (Hirota *et al*., 2012). This interaction between floral traits and pollinators not only enhances seed production but also introduces genetic diversity, which is critical for breeding programs aimed at improving disease resistance, drought tolerance, and overall adaptability of the plant (Chandra, 2013). Consequently, understanding and selecting these floral traits in cultivation could optimize both pollinator efficiency and seed yield, which are central goals in *Stylosanthes* breeding programs.

Conclusion

To the best of our knowledge, the present study is one of the few reports on uncovering flower phenological events in *Stylosanthes* species. The present study will not only facilitate a better understanding of floral biology but also provide clues for its role in the reproductive fitness of *Stylosanthes* species. However, the relationship between flower longevity and its effect on reproductive success, such as seed shattering, was not considered here.

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