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ORIGINAL PAPER

Stigma development and receptivity of two *Kalanchoë blossfeldiana* cultivars

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Abstract Several members of the *Kalanchoë* genus are popular as ornamental plants. Cross-breeding and wide hybridisation are essential to continuously introduce novel traits into cultivated plant material. This study aimed to identify the major factors related to the stigma affecting cross-pollination in the *Kalanchoë blossfeldiana.* Pollen tube growth after pollination of *K. blossfeldiana* 'Jackie' and 'Reese' was examined at different stigma developmental stages. Five distinct developmental stages were identified based on changes in morphology and activity of stigmatic peroxidase. After reciprocal pollination at the five stigma developmental stages, fluorescence microscopy was used to estimate the number of pollen tubes in situ. Both cultivars had receptive stigmas from stage I to IV, which concurred with the continuous expansion of the stigma covered with exudates. No pollen tube growth was observed at stage V for both cultivars. The number of pollen tubes was significantly higher in carpels pollinated at stage III, characterized by loose arrangement of the papillae and maximal amount of exudates, compared to all other developmental stages. Stigmas showing drying exudates and absence of peroxidase exhibited a relatively decreased number of pollen tubes in situ. No pollen tubes germinated on wilting stigmas. The arrangement of the papillae, the presence of exudates and peroxidase activity affected the number of pollen tubes in cross-pollination of *K. blossfeldiana* cultivars 'Jackie' and 'Reese'. These results will help breeders to better select the optimal time for effective pollination. The findings may be applicable for other cultivars of *K. blossfeldiana* and relevant for different species of *Kalanchoë*.

Keywords Cross-breeding · Exudates · Papillae · Peroxidase · Pollen tubes · Stigma morphology

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Introduction

Kalanchoë is a large genus of angiosperms including more than 140 species, divided into two sections *Kalanchoë* and *Bryophyllum* (Descoings 2003). Cultivars of *Kalanchoë blossfeldiana* are widely used as indoor and outdoor ornamental plants and rank among the most sold potted plants in many European countries. In 2011, 78 million plants were produced in the Netherlands with a turnover of 54 million euro (Floraholland 2013). In Denmark, *Kalanchoë* was the most important potted plant with 43.8 million plants sold in 2011 (Floradania Marketing 2013).

Most commercial cultivars are the result of cross-breeding from *K. blossfeldiana* selecting dwarf potted plants with little variability except for flower characteristics (Izumikawa et al. 2008; Mii 2009). Breeders have tried to increase the variability in traits by attempting a variety of intra- and inter-specific crosses using wild species as genetic resources, but so far with limited success. However, successful examples are the cultivars 'Wendy' and 'Tessa', suitable for creeping and hanging uses, bred from intra-sectional crosses within the *Bryophyllum* section (Izumikawa et al. 2008).

Hence, improvement of existing cultivars is necessary for plant breeders to remain attractive on competitive markets. It is desirable to improve certain characteristics of the *Kalanchoë* genus, by crossing *K. blossfeldiana* cultivars with a wide range of wild species possessing unique characteristics such as attractive leaf shape and colors, different branching habit, different shape of inflorescences, cylindrical flower shape and pendent flower shape (Mii 2009).

Consequently, to ensure product development in competitive breeding companies, exploration of novelties and improvement of existing cultivars is necessary to maintain genetic improvement through techniques such as cross-breeding or gene transformation. Only a few recent studies have investigated crossability and cross-compatibility in intra-sectional crosses in the *Kalanchoë* section and interspecific crosses between the *Kalanchoë* section and the *Bryophyllum* section (Mii 2009). Cross-breeding of *K. blossfeldiana* cultivars and wide hybridisation are frequently used in breeding programs to produce improved varieties and increase variability in ornamental traits. Although cross-breeding is widely used in *Kalanchoë,* there is still a lack of research about the influence of stigma development on pollination processes.

Effective pollination is closely linked with the duration of stigmatic receptivity, i.e., ability of the stigma to support adhesion and germination of compatible pollen (Sanzol and Herrero 2001). Several studies have focused on the factors influencing the efficiency of pollination processes. Earlier studies have documented a correlation between flower age at pollination and stigma receptivity, fruit set and the number of seeds per fruit in other plant species such as *Vaccinium ashei*, *Symphytum officinale*, *Prunus dulcis* and several species of *Dianthus* (Brevis and NeSmith 2006; Masierowska and Stpiczynska 2005; Yi et al. 2006; Fu et al. 2011). However, knowledge on the optimal stigma development for efficient pollination of *Kalanchoë* is still limited.

The purpose of the present study was to identify the major factors associated with the stigma that influence the pollination process of *K. blossfeldiana*. We evaluated the pollen viability and germinability of the two cultivars 'Jackie' and 'Reese' used in cross-pollination. We described the morphological changes that characterized the development of *K. blossfeldiana'*s stigma following flower opening and monitored the presence of peroxidase on the stigma surface to determine stigma receptivity. Finally, we assessed pollen tube growth in situ using fluorescence microscopy.

Materials and methods

Plant material and sexual hybridisation

Two cultivars of *K. blossfeldiana* 'Jackie' and 'Reese' obtained from Knud Jepsen A/S (Hinnerup, Denmark) were used in the experiments. Plants were grown in the greenhouse under natural light and temperature of 18–22 °C. Reciprocal crosses were conducted in spring and summer 2012. Flowers of the maternal parents were emasculated on the day of flower opening, by removing both stamens and petals. During the 16 days following flower opening, the stigmas were hand-pollinated once with pollen from four stamens of two randomly selected flowers, which had opened on the day the pollen was collected.

Pollen analysis

Pollen viability was determined at the day of flower opening by staining with 1 % (w/v) acetocarmine solution. Pollen germinability in vitro was assessed by culturing pollen grains in liquid medium [10 g/L sucrose, 0.01 % (w/v) H_3BO_3, 0.01 % (w/v) KH_2PO_4, 0.01 % (w/v) $CaCl_2$ and 0.02 % (w/v) $MgSO_4{\cdot}7H_2O$] at room temperature in darkness for 2 h. At least 400 pollen grains were analyzed per cultivar. The selection of culture medium was made following a preliminary screen in which different concentrations of sucrose and boric acid were tested.

Observation of stigma development

The morphological changes of the stigmatic tissue were monitored during the 16 subsequent days after flower opening using a stereomicroscope (Leica MZ12, Leica, Wetzlar, Germany) equipped with a digital camera (Leica DFC420, Leica, Wetzlar, Germany). 480 pistils were analyzed per cultivar (i.e., 30 pistils per day).

Histochemical test for presence of peroxidase

The peroxidase activity was examined on non-pollinated stigma surfaces. A modified version of Dafni and Maués' (1998) protocol was followed using the enzymatic Peroxtesmo Ko indicator paper test (Macherey-Nagel, Düren, Germany). The indicator was dipped in a drop of water and applied on top of the stigma. 480 pistils were analyzed per cultivar (i.e., 30 pistils per day).

Pollen tube growth in situ

Pollen tube growth was monitored during the 16 subsequent days after flower opening using a modified protocol of Martin (1958). The pistils were fixed in absolute ethanol and glacial acetic acid (3:1) for 24 h and stored in 70 % ethanol at 5 °C until use. The pistils were washed with distilled water before being softened in 1 M NaOH at 55 °C for 25 min and washed and stained in decolorized 0.1 % (w/v) aniline blue (in 0.1 M K_3PO_4) for 24 h in a lightproof box. The pistils were squashed in a drop of 40 %

glycerol and viewed under fluorescence microscope (Leitz DMRD, Leica, Wetzlar, Germany). At least 240 pistils, 5–7 per day, were analyzed per cultivar.

Statistical analysis

Data were analyzed using the Microsoft Excel package. Significant differences were calculated using Student *t* tests at $p \leq 0.05$ for pollen viability and germinability and at $p \leq 0.01$ for pollen tube growth in situ.

Results

Pollen analysis

The percentage of pollen viability for *K. blossfeldiana* 'Jackie' was 57 ± 3 % and significantly lower than for 'Reese' with 82.3 ± 2 %. Pollen germinability in vitro showed the same pattern with levels of 23.7 ± 2 % and for 'Jackie' and 44 ± 5 % for 'Reese'. Moreover, the different methods revealed significantly higher pollen viability than germinability for both cultivars (Fig. 1).

Stigma developmental stages

Distinct morphological changes of the stigma were observed during the subsequent 16 days after flower opening. Five stages were defined based on the expansion of the papillae and presence of peroxidase activity. The five distinct morphological stages were named: I, smooth stage; II, pre-expanded stage; III, sticky stage; IV, brown coat stage and V, wilting stage (Table 1; Fig. 2). Although both cultivars displayed the same changes, they occurred at different days after flower opening for each cultivar (Table 1).

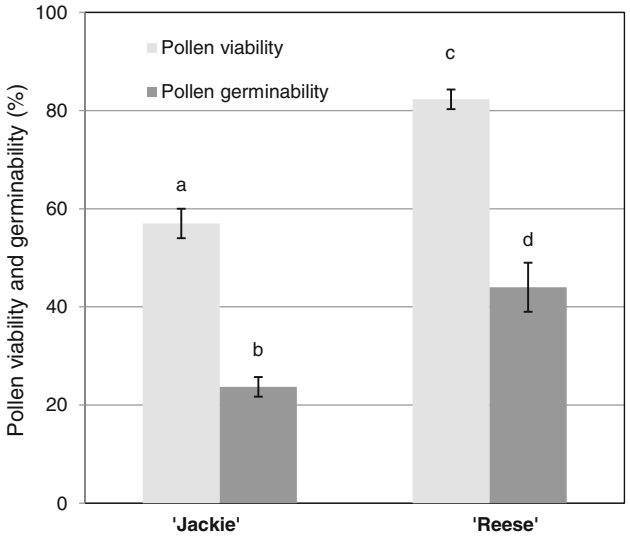

Fig. 1 Viability and germinability of pollen from *K. blossfeldiana* 'Jackie' and 'Reese'. The *bars* indicate the mean values (±SE) in percentage obtained from 3 replicates. *Values* followed by *different letters* are significantly different at $p \leq 0.05$

Pollen tube growth in situ

Pollen tube growth varied at the different developmental stages of the stigma. The analysis of the number of pollen tubes in each carpel was based on three indices: 0, no pollen tubes; 1, <10 pollen tubes and 2, >10 pollen tubes.

At stage I, <10 % of the carpels contained pollen tubes and each carpel had less than 10 pollen tubes for both of the cultivars (Table 2). At stage II, 34 % of carpels analyzed for 'Jackie' and 15 % of carpels analyzed for 'Reese' contained pollen tubes. Moreover, for 'Jackie' 4 % of the carpels contained more than 10 pollen tubes. However, the number of pollen tubes was <10 in all carpels containing pollen tubes for 'Reese' (Table 2). At stage III, the maximum number of carpels showing presence of pollen tubes was found, compared to all other stages, with 54 % carpels for 'Jackie' and 39 % carpels for 'Reese' (Table 2). In addition, the maximum number of carpels containing more than 10 pollen tubes, i.e., 9 % of total carpel number for 'Jackie' and 18 % for 'Reese' was also observed at stage III. At stage IV, a decreased number of carpels containing pollen tubes was observed, with 8 % carpels for 'Jackie' and 1 % carpels for 'Reese'. For both cultivars, most of the carpels had <10 pollen tubes. No pollen tubes were observed in carpels, when the stigma was pollinated at the wilting stage (stage V). All the stages were significantly different from stage III, the stage showing the maximum number of pollen tubes (Fig. 3).

Viable seeds were only obtained after pollination of flowers with stigmas in the receptive stage. The number of germinated plants, however, was low with only 12 plants obtained from 50 flowers when 'Jackie' was used as a mother plant, and 7 plants out of 28 flowers obtained for 'Reese' (data not shown).

Discussion

Stigma receptivity is a critical stage in the maturation of flowers and it significantly influences pollination and fertilisation success. In general, stigma is receptive to pollen for a limited time period, thus timing of pollination can be critical. Pollination outside the frame of sigma receptivity may influence compatibility of crosses and result in reduced seed set (Dafni and Maués 1998; McInnis et al. 2006a). The receptive period of stigma can vary depending on species and even cultivars. The stigma was reported to

Table 1 Five stages of stigma development of *K. blossfeldiana* 'Jackie' and 'Reese' based on changes of the stigma morphology and peroxidase activity

Stage and its name	Day of flower opening		Stigma morphological changes	Percentage of stigmas showing peroxidase activity	
	'Jackie'	'Reese'		'Jackie' (%)	'Reese' (%)
I: Smooth stage	1	1	Greenish-white stigma with a smooth surface lacking exudates	0	0
II: Pre-expanded stage	2–3	2–3	Slightly expanded stigma with more loosely arranged papillae cells, yellow to orange coloration of the stigma surface and the upper part of style. Presence of small amounts of exudates	95	95
III: Sticky stage	4–8	4–7	Expanded stigma with loosely arranged papillae and the presence of exudates with often visible drops covering the stigma. In 'Jackie', a red pigmentation visible on the top of stigma	99	100
IV: Brown coat stage	9–14	8–15	Expanded stigma covered with a brown coat of drying exudates	85	97
V: Wilting stage	15–16	16	Brown dry stigma, with signs of wilting and lack of exudates	5	17

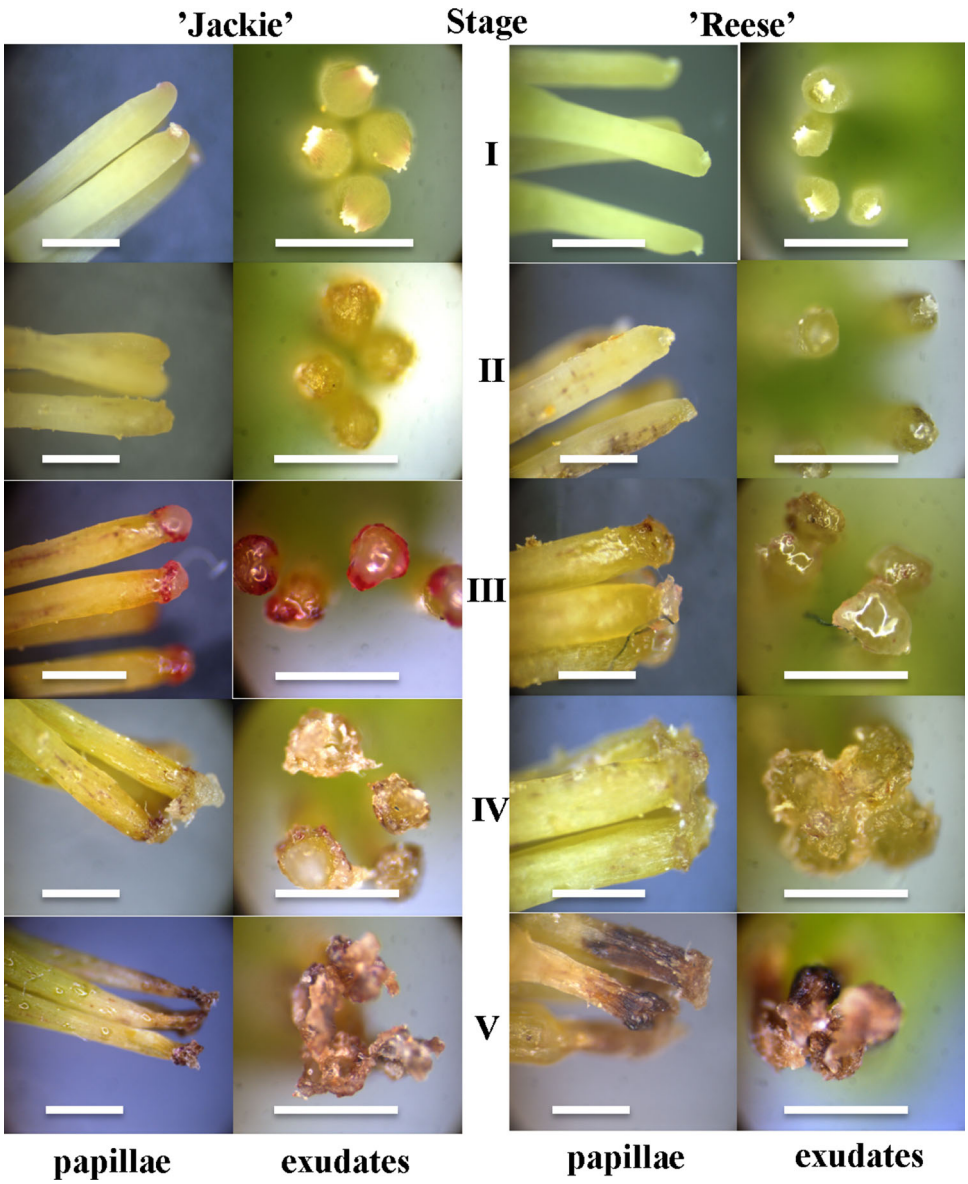

Fig. 2 The appearance of five stages of stigma development of *K. blossfeldiana* cultivars 'Jackie' and 'Reese', as seen during the 16 subsequent days after flower opening. I: smooth stage, II: pre-expanded stage, III: sticky stage, IV: brown coat stage and V: wilting stage. *Scale bars* 0.5 mm

be receptive at the time of anthesis for a number of trees like apple, apricot, sweet cherry (Yi et al. 2006), durian (Honsho et al. 2007) or wild ray (Huang et al. 2004). The receptive period of the stigma can also be delayed occurring after anthesis as it was demonstrated for carnation (Fu et al. 2011) and almond (Yi et al. 2006). In the present study, stigma receptivity for two cultivars of *K. blossfeldiana* took place between 4 and 7 days after anthesis, with both higher percentages of pollen tube germination (Table 2) and pistils exhibiting peroxidase activity (Table 1). Our data suggest that flowers in younger and older stages do not fully support pollen tube germination (Fig. 3; Table 2), whereby cross-compatibility is reduced. A number of previous studies (Egea and Burgos 1992; Young and Gravitz 2002; Souza et al. 2004; Honsho et al. 2007; Cuevas et al. 2009) focused solely on how the age of the flowers influenced stigma receptivity and pollen tube growth. These studies disregarded the influence of varying environmental factors on the rate of the flower development. Here, we addressed this issue by monitoring daily changes of the stigma and grouped into different developmental stages based on the morphological changes and the presence of stigmatic peroxidase. Other studies used similar approaches; Fu et al. (2011) investigated the influence of different morphological stages in *Dianthus* spp. on the level of pollen germination, pollen tube growth and final seed set. The stigma morphology included extension, curvation, pigmentation and wilting of the papillae cells. Similarly, the study on stigma development and receptivity in almond distinguished seven developmental stages based on the appearance of buds and flowers that coincide with stigma receptivity (Yi et al. 2006).

Table 2 Pollen tube growth at five stages of stigma development of *K. blossfeldiana* 'Jackie' and 'Reese'

Pollen tube index	Stage I (%)	Stage II (%)	Stage III (%)	Stage IV (%)	Stage V (%)
'Jackie'					
2: >10 pollen tubes	0	4	9	3	0
1: <10 pollen tubes	9	30	45	5	0
0: No pollen tubes	91	66	46	92	100
'Reese'					
2: >10 pollen tubes	0	0	18	0	0
1: <10 pollen tubes	8	15	21	1	0
0: No pollen tubes	92	85	61	99	100

In other plant species, the expansion and degeneration of papillae (Fu et al. 2011), production of exudate (Yi et al. 2006) and high enzymatic activity on stigma have been related to the time of stigma receptivity. According to Sanchez et al. (2004), the cuticle of receptive stigma is disrupted by the presence of exudates. These exudates contain cell degrading enzymes and combined with the increased turgidity of the stigma papillae growth of pollen tubes into the style is facilitated. A number of studies have shown that exudates of wet stigmas contribute to pollen capture, adhesion and hydration (Gao et al. 2010). It was also suggested that plants with wet stigma surfaces have indiscriminate adhesion that relies only on liquid surface tension (Swanson et al. 2004). Masierowska and Stpiczynska (2005) suggested that a loose arrangement of the papillae cells promotes contact of pollen grains with the

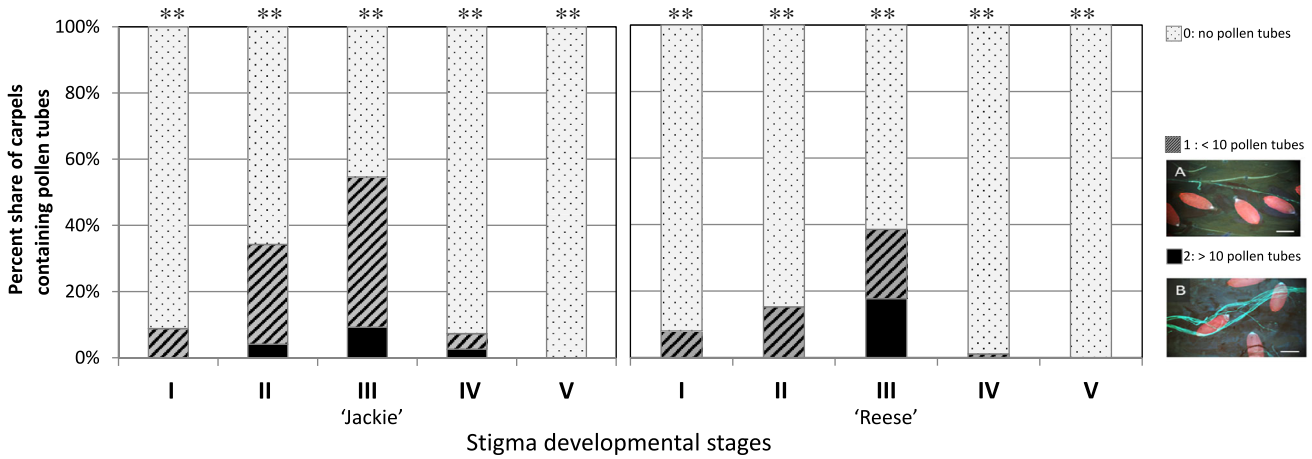

Fig. 3 Percentage of carpels with pollen tubes in each stage of stigma development. *Left K. blossfeldiana* 'Jackie' after pollination with *K. blossfeldiana* 'Reese'; *Right K. blossfeldiana* 'Reese' after pollination with *K. blossfeldiana* 'Jackie'; The amount of pollen tubes was measured in 3 qualitative indexes: 0, no pollen tubes; 1, <10 pollen tubes; 2, >10 pollens tubes. Stages significantly different from stage III ($p \leq 0.01$) are indicated by *two asterisks*. **a** Aniline blue stained pollen tubes in the ovary of *K. blossfeldiana* 'Reese' 24 h after pollination with *K. blossfeldiana* 'Jackie'; qualitative index 1: <10 pollen tubes. **b** Aniline blue stained pollen tubes in the ovary of *K. blossfeldiana* 'Jackie' 24 h after pollination with *K. blossfeldiana* 'Reese'; qualitative index 2: >10 pollen tubes. *Scale bars* 200 µm

receptive stigma, thereby facilitating pollen hydration and germination. In addition, wet stigmas release viscous surface secretion composed of proteins, lipids, polysaccharides and pigments (Edlund et al. 2004; Martin 1969), which are assumed to function as pollen tube nutrition and enable its recognition and guidance (Sanchez et al. 2004). Our results indicate that germination of pollen tubes is facilitated in stigmas with loosely arranged papillae cells and abundant coverage of exudates. The microscopic observations revealed a low amount of pollen on the stigmas where presence of exudate was limited. A small amount of stigmatic secretion in younger developmental stages suggests that pistils of *K. blossfeldiana,* similarly to other plant species with wet stigmas (Yi et al. 2006), gain competence to provide a proper environment for pollen hydration and germination. In our study, two cultivars of *K. blossfeldiana* 'Jackie' and 'Reese' were selected. Under the fluorescence microscopy we did not observe any abnormalities in the germination or growth of pollen tubes. Germinated pollen grains deposited on the stigmatic surface had no difficulty in penetrating the stigma. No abnormal callose deposition was observed in pollen tubes during the cross-pollination between cultivars (Figs. 2, 3; Table 2).

Several studies have shown that the stigmas of angiosperms exhibit high levels of peroxidase activity at maturity when they are receptive to pollen (Dafni and Maués 1998; Stpiczynska 2003; McInnis et al. 2006b). This phenomenon is used to assess the time of stigma receptivity for breeding purposes. The exact function of the enzyme, however, is still not known (McInnis et al. 2006a, b). The cell-specific expression and localization of a stigma-specific class III peroxidase (SSP) at the cytoplasmic regions and surface of the stigmatic papillae suggest that it may have a role in stigma function (McInnis et al. 2006b). With respect to pollen–stigma interaction, the role of stigma peroxidase could be to facilitate loosening of the stigma cell wall components to ease pollen tube penetration and growth within the stigma. Alternatively, it may be assumed that peroxidase is acting indirectly through H_2O_2 metabolism as a component of signaling systems, mediating species-specific pollen recognition (McInnis et al. 2006b). It was also suggested that stigmatic peroxidase may be involved in defense against pathogen attack. Since increased expression of peroxidase genes is known to be associated with hypersensitive response and stress, the high levels of peroxidase activity in stigmas may contribute to enhanced protection against pathogens attacks at maturity (McInnis et al. 2006a).

Quality of pollen is an important factor that influences cross-breeding processes. Low quality of pollen can increase the risk of pollination failure, and by that, reduce fertilisation success (Wilcock and Neiland 2002). In our study, we used two methods to assess pollen quality, i.e., acetocarmine staining and in vitro pollen germination. The differences in the results obtained by two methods were highly significant. Acetocarmine is one of the most common nuclear stains that indicate presence of cytoplasm. It was, however, demonstrated that it is not able to distinguish between fresh and thermally inactivated pollen (Rodriguez-Riano and Dafni 2000). Thus, in the present study, it can be assumed that pollen viability was overestimated by acetocarmine staining. In vitro pollen germination was preceded by preliminary screen of different sucrose and boric acid concentrations to assure optimal medium composition. Hence, pollen germinability is a more accurate measurement of the quality of pollen used in our experiment (Fig. 1).

In our study, the number of germinated plants was very low. Only 12 plants were obtained from 50 flowers when 'Jackie' was used as a mother plant, and 7 plants out of 28 flowers were obtained for 'Reese' (data not shown). However, the results of in vitro pollen germination indicated low pollen quality available at the time of pollination (Fig. 1). Thus, it is reasonable to assume that this was a critical factor that affected the number of pollen tubes observed after pollination and the final seed set in our experiment.

As suggested by de Graaf et al. (2001), the initial pollen tube growth is an autonomous process and does not depend on components produced by the female tissues. Thus, low pollen quality is likely to impair the kinetics of pollen tube growth. Together with short longevity of ovules, this factor can strongly influence the effective pollination period.

Our study indicates that in the tested cultivars of *K. blossfeldiana*, the receptive stage of stigma was delayed as it occurred after anthesis. Morphological evaluations revealed that the receptive stage is characterized by loose arrangement of the papillae and maximum presence of exudates. It also coincides with high activity of stigmatic peroxidase. These results enable breeders to find the optimal conditions for pollination of *K. blossfeldiana* cultivars by monitoring changes in stigma development. Moreover, a transfer of the obtained knowledge to other *Kalanchoë* species might be possible, since flower and stigma morphology is highly conserved within the *Kalanchoë* genus.

Author contribution L. T. Traoré did experimental design, hybridization, pollen analysis, stigma receptivity analysis, fluorescence microscopy, preparation, data analysis, manuscript writing. K. Kuligowska did experimental design, fluorescence microscopy analysis, data analysis, manuscript writing. H. Lütken did experimental design, manuscript writing and planning. R. Müller did experimental design, manuscript writing and planning.

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Conflict of interest The authors declare that they have no conflict of interest.

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