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Pollen–pistil interactions in divergent wide crosses lead to spatial and temporal pre-fertilization reproductive barrier in flax (*Linum usitatissimum* L.)

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Linseed, has been a source of natural fiber for textile industries since its domestication. However, despite being the potential source of trait reservoir, the use of *Linum* wild genetic resources for the improvement of economic traits are not exploited widely. This is mainly due to the degree of genetic divergence that exists among the interspecific ecotypes causing crossability issues. Self-incompatibility due to the occurrence of heterostyly is very well reported in distantly related crop wild relatives of *Linum* and, the mechanism of self-incompatibility between different floral morphs is also studied. However, pollen germination and tube growth responses in the interspecific crosses are rarely studied. Thus, the present study was exclusively carried out to assess the major pre-zygotic barriers and their effect on pollen germination on foreign stigma using fluorescent microscopy of aniline blue stain-aided technology, to understand how the species barriers operate on pollen germination and pollen tube growth. The study revealed that the pollen-pistil interaction in the wide crosses among *L. usitatissimum* X *L. grandiflorum* was regulated by both temporal and spatial pre-fertilization barriers. Callose deposition within 2 h after pollination (HAP) at the stigma surface, was the major cause inhibiting pollen germination. Various kinds of aberrations started appearing during the 2–4 HAP. The complexity of interspecific hybridization was observed in terms of arrest of pollen tube (PT) growth in the ovary, ruptured, twisted and swollen pollen tube tip, tube growth in reverse direction, convoluted and terminated growth patterns. Inconsistent growth rates of pollen tubes to reach various stylar regions emphasizes the importance of studying these wild relatives for potential agricultural advancements. The results show that while distant hybridization with *L. grandiflorum* is less efficient, pollen tubes can still navigate the ovular tissues, albeit with some delay. This finding opens avenues for investigating factors that hinder viable seed formation, enhancing our understanding of reproductive success in distant hybridization with this species.

Keywords Pollen-pistil interaction, *Linum*, Interspecific, Pre-fertilization barriers

Flaxseed, also known as linseed has evolved as one of the predominant industrial crops during the last few decades[1] and originally domesticated for its oilseed and natural fiber usage[2]. Grown for dual purpose, the two separate main directions have been developed for utilizing the plant's products, leading to the cultivation of fiber flax and oil flax (linseed)[3]. A rich and interesting history surrounds flax. The Fertile Crescent region, saw the domestication of this plant approximately 8,000 BCE[4] first for its seeds and later for its fibres[5]. It's fascinating that its usage existed even before domestication. Caucasus area was investigated to report flax yarn residues dated 30,000 years ago[6]. Nonetheless, the cultivation of flax as a crop for textile fiber was made possible by Ancient Egypt[7,8] highlighted the Indian subcontinent, Abyssinia, and the Mediterranean area as major regions

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of flax diversity. In these regions, the wild ancestor *Linum bienne* was cultivated, likely in isolation, leading to the domestication of *L. usitatissimum*. Thus, these areas could be associated with multiple domestication events of flax. These days, high-end fabrics, natural actuators[9], and composite material reinforcements[10] all use flax fibres. Therefore, flax is a link between cultures and periods[11]. As a vital industrial resource, linseed oil is employed for multiple purposes, primarily as a fundamental component in paints, resins, printing inks, varnishes, and linoleum[12]. Advances in material science have brought fiber flax back into the spotlight, as its fibers are now being harnessed for a multitude of environmentally sustainable industrial uses, such as composites, geotextiles, insulation, and specialty papers. In India during the winter season, linseed is the second most important oilseed crop after rapeseed mustard in terms of both area and production. It holds the top position in the country for industrial oil production and holds the top spot globally in terms of acreage, representing 23.8% of the world total, and ranks third in production, accounting for 10.2% of the world total[13]. Every part of the linseed plant is commercially utilized, either directly or after processing. The seed contains 33 to 47% of oil, containing around 58% omega-3 fatty acid. Flax is cultivated on an area of about 2.31 million hectares globally. Asian countries contribute about 49.2% area in the world[14]. India, Canada, China, the USA, and Ethiopia are the main countries for linseed production.

The existence of modern-day flax varieties mainly includes domestication and selection as important historical breeding phenomenon. The initial bred flax varieties to surface in Southern Europe, especially in the Danube valley, were winter oil crops[15]. Evidence from history shows that bast fiber textiles extended to the Nile Valley and even to present-day Britain by 4,000 BC. In Eastern Europe, the creation of spring-planted fiber varieties is thought to have originated from either a unique domestication event in Central Asia (Indo-Afghan region) or from the existing European varieties found in the south[16,17]. At the dawn of the 20th century, Russia established itself as the foremost supplier of top-quality fiber flax to Europe. The tireless efforts of local peasants in cultivation gave rise to "kryazh," a set of heritage landraces that developed over centuries through both natural and artificial selection, focusing on the creation of tall fiber flax varieties. These kryazh varieties were considered premium commodity crops, leading to their active trade and incorporation into breeding programs worldwide. It is widely recognized that the origins of all modern fiber flax varieties can be traced back to Eastern Europe[18].

Flaxseed, being a self-pollinated crop, has faced the narrowing down of its genetic base due to selection during domestication and plant breeding just like all other cultivated crops[19]. The wild progenitor species gave rise to all the major crops around the world, mainly about 4,000 to 10,000 years ago through domestication[20]. New variants can be derived from wild relatives or intermediary landraces, and a portion of these can be beneficial for enhancing crops through traditional breeding techniques or biotechnology. *Linum* is one of the largest genera of *Linaceae* family, containing more than 200 species[21] growing in diverse geographical regions of world. In India, apart from cultivated flax viz., *L. usitatissimum*, the occurrence of other five Crop Wild Relatives (CWR's) namely, *L. strictrum*, *L. bienne*, *L. grandiflorum*, *L. mysorense* and *L. perenne*[22] is reported. However, utilization of wild linseed species in crop improvement programs has been limited partly because of lack of collection and conservation of diverse species in gene banks and partly because of the degree of genetic divergence that exists among the interspecific ecotypes causing crossability issues.

Large number of CWR's of flax are available worldwide in genus *Linum* that could be of immense potential for their inclusion as trait donors under various flax improvement programs. However, the genus *Linum* has many diploid species that exhibit a remarkable diversity in chromosome number including $n = 8$, 9, 10, 12, 14, 15, 16, 18, 30, and > 30[23,24]. No absolute studies have yet emerged as a reflective of phylogenetic relationships among *Linum*species. There are uncertainties regarding the chromosome numbers of some wild species. The differences in chromosome size and their number pose immense hurdles in hybridizing CWRs with cultivated flax. These genetic differences between wild and cultivated species have their unfavorable impact on feasibility of hybridization among flax wild species in both or at least one direction. Successful production of fertile hybrids to an extent, is assumed to occur only in species with equal chromosome numbers. A comprehensive detail on this aspect has been represented by[21].

Potential usage of some wild relatives of *Linum* includes fiber quality and core hardness (*L. bienne*), bud fly resistance, lignan and neolignans synthesis (*L. grandiflorum*), nutritious and oily quality (*L. lewisii*), drought and rust tolerance (*L. marginale*). Despite their huge potential, the use of these CWR's under flax improvement programs is very scarce. Studies focused on the crossability potential[25]; Jhala et al[21]. or elaborating the reasons behind the reproductive isolation in flax are limited. Interspecific hybridization between wild and cultivated species can play a major part to add economically important characters in *Linum*.

Pollen-pistil interaction (PPI) offers enormous potential for the manipulation of pollen screening which is obviously for the compatibility of pollen. PPI can be considered as an important contributory factor in introducing genetic diversity to the flowering plants from their wild and weedy counterparts. Assessing the compatibility of foreign pollen on cultivated species during pollination and fertilization offers enormous potential to study the PPI and understand incompatibility barriers[26–28]. This may broaden the scope for bringing wild species under crop breeding programs for the improvement of traits of interests or introducing genetic diversity.

Self-incompatibility due to the occurrence of heterostyly is very well reported in different species related to cultivated *Linum*[29–31]. Also, the mechanism of self-incompatibility between different floral morphs has been investigated[32–34]. However, to the best of our knowledge, there is rarely any reference literature available pertaining to germination and tube growth of foreign pollen grain onto the stigmatic surface and stylar regions of the parent belonging to another species in *Linum*. Despite many attempts for making wide crosses in *L. usitatissimum* with its distant relative i.e., *L. grandiflorum*, we had been unsuccessful getting any viable seeds under field conditions. Therefore, the present investigation was carried out to (i) assess the major pre-zygotic barriers and their effect on the germination of pollen grains on foreign stigma using fluorescent microscopy of aniline blue stain aided technology, (ii) understand how the species barriers operate on pollen germination (PG)

Scientific Name	*L. usitatissimum* L.	*L. bienne* Mill.	*L. grandiflorum* L.
Common name	Flax/linseed	Pale flax	Red flax/ Scarlet flax
Biological Status	Germplasm (Registered genetic stock)	Wild progenitor	Distant CWR
Heterostyly	Homostylic	Homostylic	Distylic
Self-compatibility	Self-compatible	Self-compatible	Relatively incompatible
National ID	IC268345	EC993391	IC633096
Chromosome number (2n)	30	30	16
Potential use	High oil content (42.5%)	Fibre quality and more primary branches	Bud fly resistance
Growth habit	Bushy to erect	Prostrate growth habit	Semi-spreading
Cross compatibility with cultivated species	Yes	Yes	No (Shriveled seeds)
Geographic distribution/origin	Southern Europe, the Near East, or Central Asia	Mediterranean Sea, Iran, and the Canary Islands	Native to Algeria but occurrence is found in India, Northern Africa, Southern Europe and North America
Procured from	National Genebank, ICAR-NBPGR, New Delhi, India	CGN Gene Bank, Plant Research International Wageningen, Netherlands	National Genebank, ICAR-NBPGR, New Delhi, India

Table 1. Details of plant material used in the study.

S. No.	Nature of Cross	Species involved (pistil side X pollen donor)	Accessions
1.	Self-pollination	*L. usitatissimum* X *L. usitatissimum* (*Lu* X *Lu*)	IC268345 X IC268345
2.	Self-pollination	*L. bienne* X *L. bienne* (*Lb* X *Lb*)	EC993391X EC993391
3.	Self-pollination	*L. grandiflorum* X *L. grandiflorum* (*Lg* X *Lg*)	IC633096 X IC633096
4.	Cross-pollination	*L. usitatissimum* X *L. bienne* (*Lu* X *Lb*)	IC268345 X EC993391
5.	Cross-pollination	*L. bienne* X *L. usitatissimum* (*Lb* X *Lu*)	EC993391 X IC268345
6.	Cross-pollination	*L. usitatissimum* X *L. grandiflorum* (*Lu* X *Lg*)	IC268345 X IC633096
7.	Cross-pollination	*L. grandiflorum* X *L. usitatissimum* (*Lg* X *Lu*)	IC633096 X IC268345

Table 2. Details of cross/self-combinations attempted for wide hybridization.

and pollen tube growth (PTG) when different wild species are used as the female parents and (iii) account for the impact of maternal cytoplasm on pollen rejection, PTG, and fertilization in different cross combinations.

Materials and methods

Details of *Linum* species used in the study

Linseed accession IC268345 represented cultivated species (*L. usitatissimum* L., 2n = 30, homostylous) of *Linum* for this investigation. The wild species used under the current study involved its wild progenitor, *L. bienne* M. (2n = 30) (accession EC993391, homostylous) and the other distant CWR *L. grandiflorum* L. (2n = 16). Since, *L. grandiflorum*is distylic but unlike most distylic plants the level of the anthers is not greatly different in the two types[30–32] we used a mixed population of originally procured *L. grandiflorum* (IC633096) to ensure the legitimate PG and subsequent PTG in the pistils of manually pollinated flower buds. Details of the plant materials used and its characteristic features are listed in Table 1.

Field experiment and hand emasculation

The field experiment was conducted at Indian Council of Agricultural Research – National Bureau of Plant Genetic Resources, experimental farm, IARI, New Delhi (28° 38′ 53.7′′ N, 77° 09′ 05.4′′ E and 218 m above mean sea level) in paired row of 2 m length and row spacing of 90 cm during cropping season from November to April, 2021-22. Self and cross-pollination were attempted manually during the season using cultivated species IC268345, wild species EC993391 (*L. bienne* M.) and IC633096 (*L. grandiflorum* L.) with reciprocal cross combinations as represented in Table 2. To assure the continuous availability of flowers, all the three species were staggered planted on three sowing dates, after 3–5 days of intervals. Artificial pollination was carried out following the method suggested by Bolling et al.[35] with slight modifications. A day before pollination, the un-opened flower buds with exposed petals were hand emasculated during the evening hours, between 5 and 7 PM. For emasculation, all the five anthers were exposed for removal using forceps. The petals were left intact in the buds to allow anthesis next morning. This helped exposure of stigmas naturally avoiding any damage to the stigmas while making efforts to separate the sepals covering stigmas next morning. This slight modification also helped transferring the pollens grains on the surface of naturally exposed stigma while performing pollination manually, that otherwise needed extra efforts to keep the sepals stay apart from each other while pollen transfer. After all the five anthers were removed, the pistils were closed back with petals and bagged with waxy pollination bags to avoid drying up of stigmas. Bagging of donor flower buds was also done the previous evening to obtain fresh non-contaminated pollen grains to hybridize the emasculated flower buds the next morning to avoid cross contamination. The next morning between 7 and 9 AM, the pollen was transferred using freshly opened bagged flower by directly placing the pollen grains onto the exposed stigma of emasculated flower by contact method.

Transfer of a sufficient number of pollen grains was ensured. The pollinated flowers were tagged with all the relevant information and bagged immediately to avoid cross contamination. Self-pollination was done in the same manner for all the three species.

Pollen vitality test

The vitality of pollen grains in *L. usitatissimum*, *L. bienne*, and *L. grandiflorum* was assessed using a Leica light microscope. To begin, mature flower buds were carefully collected and kept in an ice container. Subsequently, the anthers were delicately excised from the unopened bud, placed on slides, and gently crushed by dropping 1% (w/v) acetocarmine solution (Sigma-Aldrich Chemical Pvt Limited) with the aid of forceps and a needle. No incubation period was provided to stain the pollen grains. After removing any debris, a coverslip was carefully positioned on the slide before observation under the microscope. When the cytoplasm and nuclei of the pollen grains were stained in red, they were considered viable, whereas the colorless pollen grains were considered non-viable and sterile. The captured images were then utilized to quantify and evaluate the stained and unstained pollen grains by direct count. During assessment, nonviable pollen, showing signs of shriveling or remaining unstained, was distinguished from viable, round, plump, and stained pollen. Each of the species under scrutiny was thoroughly examined using four to six slides, each divided into 4 to 8 nonoverlapping sections.

Standardization of protocol for the study of pollen-pistil interaction in wide crosses of *Linum*

Sample collection and storage

The pistils of the artificially self and cross-pollinated flower buds were collected without separating them from petals and sepals after 2, 4, 6, 8, 10, 24 and 48 h after pollination (HAP) followed by fixation in acetic acid: alcohol (1:3 v/v) fixative in amber colored 5 ml glass vials and stored at 4 °C until further analysis. For every collection time, self and cross-pollinated flower buds were used for microscopic observations. For each self and cross combination 15 artificially pollinated flower buds were collected. Thus, for all the seven cross combinations of each duration, a total of 735 flower buds were collected and studied further.

Sample processing

To study PPI, aniline blue fluorescence method[36] was modified slightly for *Linum* for softening period by altering sodium hydroxide (NaOH) concentration. This helped to reduce the time duration of softening treatment for dissected pistils thereby, increasing the efficiency of sample handling with reduced time duration and minimal damage to the fixed pistils. Instead of direct fixation of pistils over the fixed flower buds, was proved advantageous in terms of keeping the fibrous *Linum*pistils retaining their integrity while sample processing without breaking the fragile tissues. The fixed pistils along with the intact flower buds were washed 2 to 3 times with distilled water for 5 min each followed by dissection. The pistils were dissected out from the flower buds with the help of pointed forceps and needles available in biological experiments dissection box to separate out the attached stigmatic regions. Unlike, Parani[36] where higher concentration of softening agent (8 N NaOH) was used for longer duration (8 h), we reduced the concentration to 6 N NaOH with 1 h time duration to clear and soften the tissue at room temperature. The softened pistils were kept undisturbed in the distilled water for 30 min followed by mild washing to avoid any damage to the softened tissue and to remove all traces of softening agent. This enhanced the absorption of fluorescent dye in the target tissue. The softened tissue was then subjected to staining buffer overnight under dark conditions for further analysis through fluorescent microscopy.

Fluorescence microscopy

For preparation of staining buffer, 0.1 M dibasic potassium phosphate (K_2HPO_4) mixed with 0.1% water-soluble aniline blue dye (WS Color Index 42780) was used to stain the processed pistils. Staining with 0.1% water-soluble aniline blue prepared in 0.1 M dibasic potassium phosphate (K_2HPO_4) overnight, gave good results in terms of dye absorption by the target tissue. The stained pistil was then placed on glass slide without glycerol treatment to avoid intertwining of fibrous pistils. Pistils were covered with cover slip and pressed gently into a thin layer. The slides were placed in dark till further observation. The image was captured under dark room condition and freely definable black balance available with the Leica DM6 B Upright Fluorescent Microscope using 390–420 nm barrier filter coupled with a 450 nm excitation filter. Pollen germination and tube growth image thus captured with a Leica DFC7000 T camera installed with the microscope was used to process the image with Leica-X Software for taking various parameters such as pollen germination and determination of PTG in different time intervals.

Results

Seed setting

No viable seeds under field conditions were obtained in the crosses involving *L. grandiflorum* X *L. usitatissimum* (*Lg X Lu*) and *Lu X Lg* (Fig. 1). During the field experiments in case of interspecific hybridization in *L. usitatissimum* and its wild progenitor i.e., *L. bienne*, the seed set was successful up to 81.54 to 83.29% including reciprocals. In the case of *L. grandiflorum*, it is concerning that either no seeds were produced at all, or the seeds that did develop were chaffy (Fig. S1) and showed no signs of embryo formation within the seed coat. This was particularly striking given the observed capsule formation rates, which varied significantly from 21.97% (*Lg X Lu*) to an impressive 70.12% (*Lu X Lg*) (Table S1). This raises important questions about the viability of *L. grandiflorum* in reproductive success.

Pollen vitality

Since, pollen vitality affects the growth of pollen tubes on the stigma, it was crucial to conduct pollen vitality test which revealed an average 69.92% vital pollen grains in *L. usitatissimum* in 3,108 studied total pollen grains.

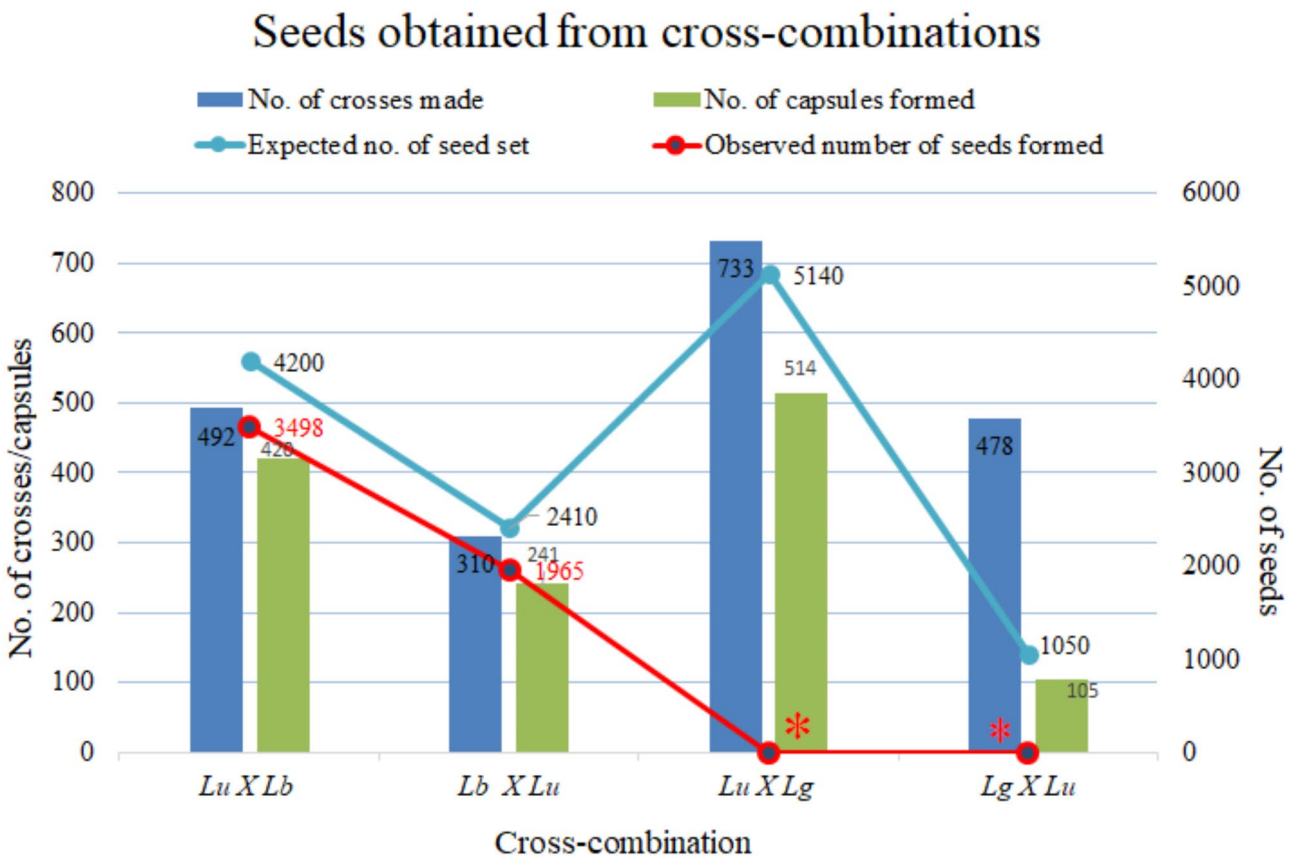


Fig. 1. Graphical representation of seeds obtained in different interspecific cross combinations attempted with its wild relatives in *Linum*. The asterisk mark signifies "no" seeds formation under field conditions for the *Lu X Lg* and *Lg X Lu* cross combinations.

In *L. bienne*, average pollen vitality percentage (PV%) was 86.35% in 1,368 studied pollen grains and in *L. grandiflorum*, the average was 91.30% (Fig. 2, Table S2). Both the wild relatives had statistically significant higher PV% ($p < 0.05\%$) compared to the cultivated *Linum* (Table S3).

Pollen germination on stigmatic surface of self-pollinated buds

Analysis of PG in self- and cross-pollinated stigmatic surfaces of processed pistils was done as the percent PG (%) for the respective duration. Overall PG in self-pollination for *L. usitatissimum* X *L. usitatissimum* (*Lu* X *Lu*) was 94.51%, for *L. bienne* X *L. bienne* (*Lb* X *Lb*) was 94.27%, for *L. grandiflorum* X *L. grandiflorum* (*Lg* X *Lg*) was 94.12% with a maximum germination recorded during 10 HAP (95.99%), 8 HAP (95.58%) and 2 HAP (94.82%) in the *Lu*, *Lb* and *Lg* respectively (Fig. 3a, b **and c**) without any significant differences ($p > 0.05\%$) among the three species (Table S3). The analysis of the captured images showed more than 90% PG on the stigmas in all the three species in case of self-pollination in each species within 2 HAP (Fig. 4a, e and i). Both the wild relatives took lesser time duration to attain maximum pollen germination percentage, compared to the cultivated *Linum*. The pollen germination and the journey of PTG to reach up to the ovule is represented in *Lu* (Fig. 4a–d), *Lb* (Fig. 4e–h) and *Lg* (Fig. 4i–l). Schematic illustrations of various regions of pistil in *Linum* is represented in Fig. 4u.

Pollen germination on stigmatic surface of cross-pollinated buds

For the cross combinations involving *L. bienne*, the PG percentage on the foreign stigmas was more than 90% in both direct and reciprocal combinations. Overall PG in cross-pollination for *Lu* X *Lb and Lb* X *Lu* was 94% and 94.87% (Table S3). The maximum PG was observed in both these combinations was found 4 HAP (97% and 96.19%) (Table S4) with 93.71% (*Lu X Lb*) (Fig. 3d) and 95.20% (*Lb X Lu*) (Fig. 3e) PG as first observation taken 2 HAP. For the cross combinations involving *L. grandiflorum*, the PG percentage on the foreign stigmas was restricted up to about 8.82% in both direct and reciprocal combinations (Table S3). Overall PG in cross-pollination for *Lu* X *Lg* and its reciprocal *Lg X Lu* was 8.15% and 8.82% for all HAP with a maximum of 10.92% (4 HAP) and 10.33% (6 HAP). For the first observation taken 2 HAP the PG percent was only 7.77% (Fig. 3f) and 8.65% (Fig. 3g) respectively for these cross combinations. In case of self-pollination, PG was significantly higher than in cross combinations. Also, the observation on PG in cross combinations of cultivated *Linum* with *Lg* were found significantly reduced ($p > 0.05\%$) compared to its wild progenitor (*Lb*) (Table S3). This indicated towards the level of incompatibility between the foreign pollen on stigmatic surface of the cultivated *Linum* species. It

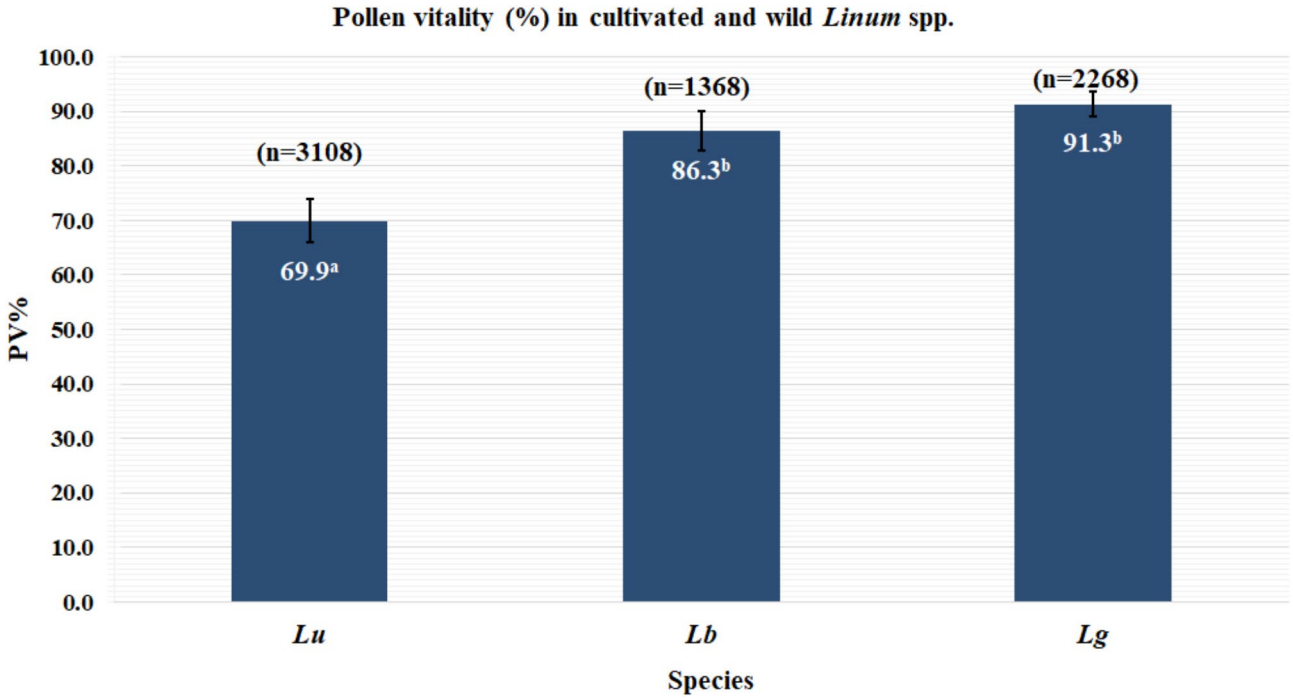


Fig. 2. Graphical representation of pollen vitality (PV%) in cultivated (*Lu*) and wild *Linum* spp. (*Lb* and *Lg*). Identical superscript letters on average PV% values for each individual species indicates that these values are statistically insignificant while, differed superscript letters indicate that the values are statistically significant ($p < 0.05$). PV% indicates pollen vitality percent. The 'n' value above each bar in brackets indicates the total number of pollen grains studied in each species. The cap-tipped error bars on each bar indicate the standard error for PV% of each species, calculated by dividing the standard deviation by the square root of the sample size. Shorter error bars with smaller caps imply higher confidence in the data point.

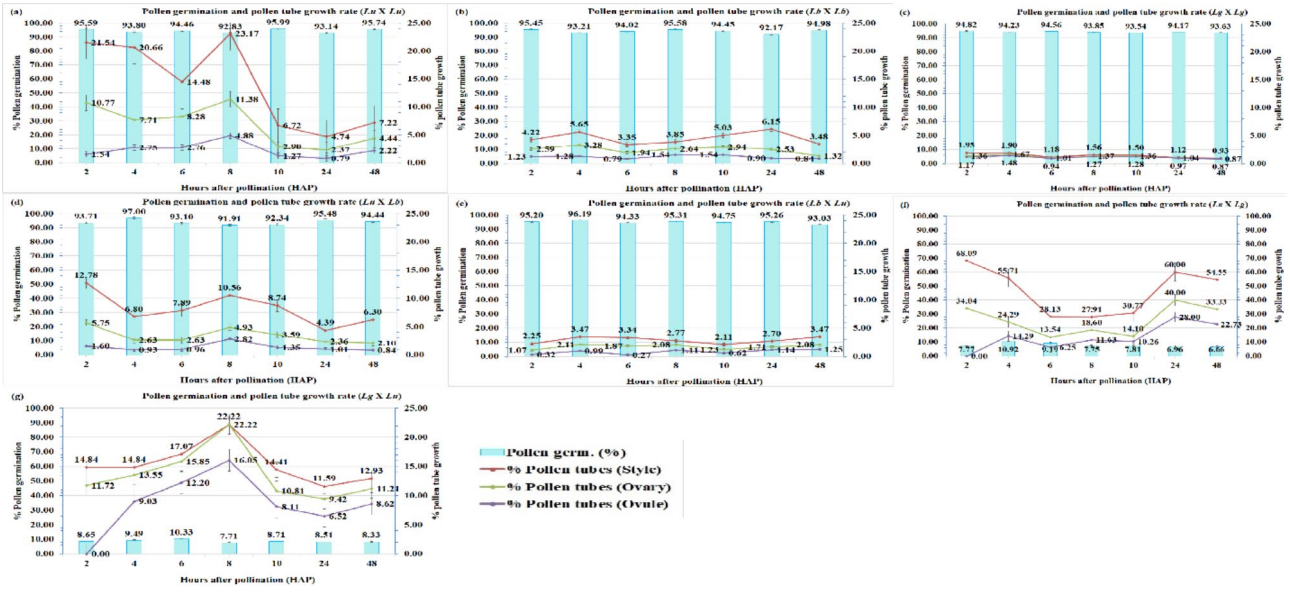


Fig. 3. Visualization of pollen germination and pollen tube growth in different hours after pollination (HAP) in various cross combinations in *Linum* spp.

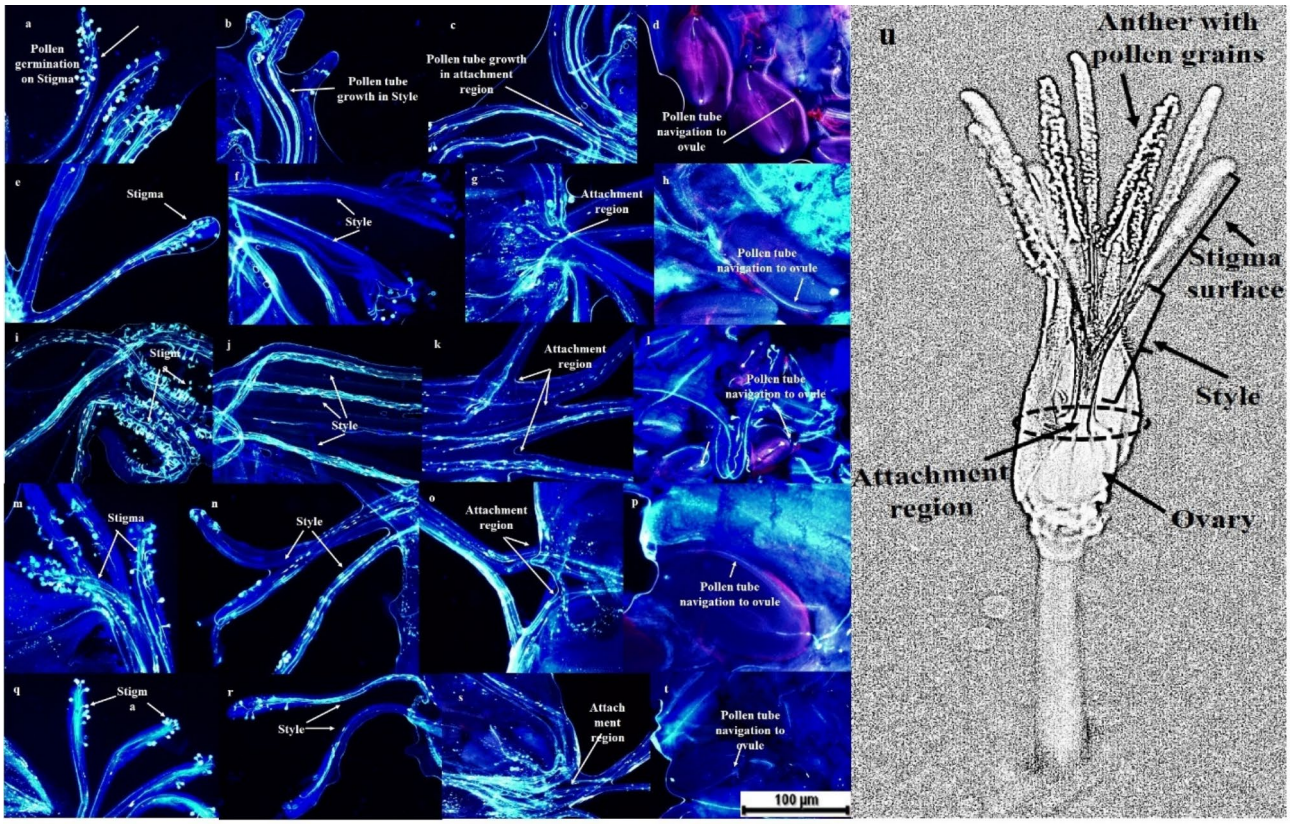


Fig. 4. Pollen germination on self-pistils of *Lu*, *Lb*, and *Lg*, plus compatible crosses between *Lu X Lb* and its reciprocal. (**a**, **b**, **c**, **d**): Pollen germination, pollen tube growth in style, PTG in attachment region and, pollen tube navigation to ovule in self-pollinated pistils of *L. usitatissimum* (2 HAP); (**e**, **f**, **g**, **h**): Pollen germination, pollen tube growth in style, PTG in attachment region and, pollen tube navigation to ovule in self-pollinated pistils of *L. bienne* (2 HAP); (**i**, **j**, **k**, **l**): Pollen germination, pollen tube growth in style, PTG in attachment region and, pollen tube navigation to ovule in self-pollinated pistils of *L. grandiflorum* (2 HAP); (**m**, **n**, **o**, **p**): Pollen germination, pollen tube growth in style, PTG in attachment region and, pollen tube navigation to ovule in pistils of *L. usitatissimum* pollinated with *L. bienne* (2 HAP); (**q**, **r**, **s**, **t**): Pollen germination, pollen tube growth in style, PTG in attachment region and, pollen tube navigation to ovule in pistils of *L. bienne* pollinated with *L. usitatissimum* (2 HAP); (u) Schematic illustrations of various regions of pistil in *Linum*.

was noteworthy that the journey of PTG from stigma to the ovular region was completed within 2 HAP in *Lu* X *Lb* (Fig. 4m–p) and *Lb* X *Lu* (Fig. 4q–t). The reduced pollen germination and deposition of callose 2 HAP, on the stigma surface in the crosses involving *Lg* as one of the distant parents is evident in Fig. 5a (*Lu* X *Lg*) and Fig. 5b (*Lg* X *Lu*). Also, the pollen grains lying ungerminated on the stigmatic surface in these crosses due to the heavy callose deposition could be seen during different hours of observations (Fig. 5c–g).

Pollen tube growth in self-pollinated pistils

In case of self-pollination in all the three species, it took only 2 HAP to grow through the stylar region and reach to up-to the ovule (Fig. 3a, b **and c**). The maximum number of pollen tubes (PTs) observed reaching to ovules (12) were in 8 HAP in *L. usitatissimum*. The germinated PTs were observed growing with different rates in pistil that was inconsistent during different hours of observation in stylar, attachment region of ovary and ovular region inside the ovary. In 2 HAP, data indicated that only 1.54% of PTs were able to penetrate the ovular region within the ovary. However, during 8 HAP, this percentage demonstrated an increase to a maximum of 4.88% (Fig. 3a). It is worth noting that the growth rates observed in the subsequent hours varied, which introduces some complexity in interpreting a definitive growth trend. For *L. bienne*, maximum number of PTs observed reaching to ovules (14) were in 4 and 8 HAP. For *Lb*, again the germinated PTs showed inconsistent growth rates in different stylar regions. During 2 HAP, 1.23% of PTs were noted to penetrate the ovular region within the ovary, whereas a maximum of only 1.54% (8 and 10 HAP) PTs could be seen penetrating ovular region inside the ovary (Fig. 3b). However, it is crucial to highlight that as observations extended into later hours, showed decline in PT growth in the ovule. In case of *L. grandiflorum*, the PTG was comparatively lower in style (10), ovary (7) and ovule (6) during the initial two hours of observation (2 HAP) compared to *Lu* and *Lb* (Table S4). The maximum number of PTs were observed only during 8 HAP in these regions. During 2 HAP, 1.17% of PTs were noted to penetrate the ovular region within the ovary, whereas a maximum of only 1.48% (4 HAP) PTs could be seen penetrating ovular region inside the ovary which again showed varied rates of PTG in the subsequent

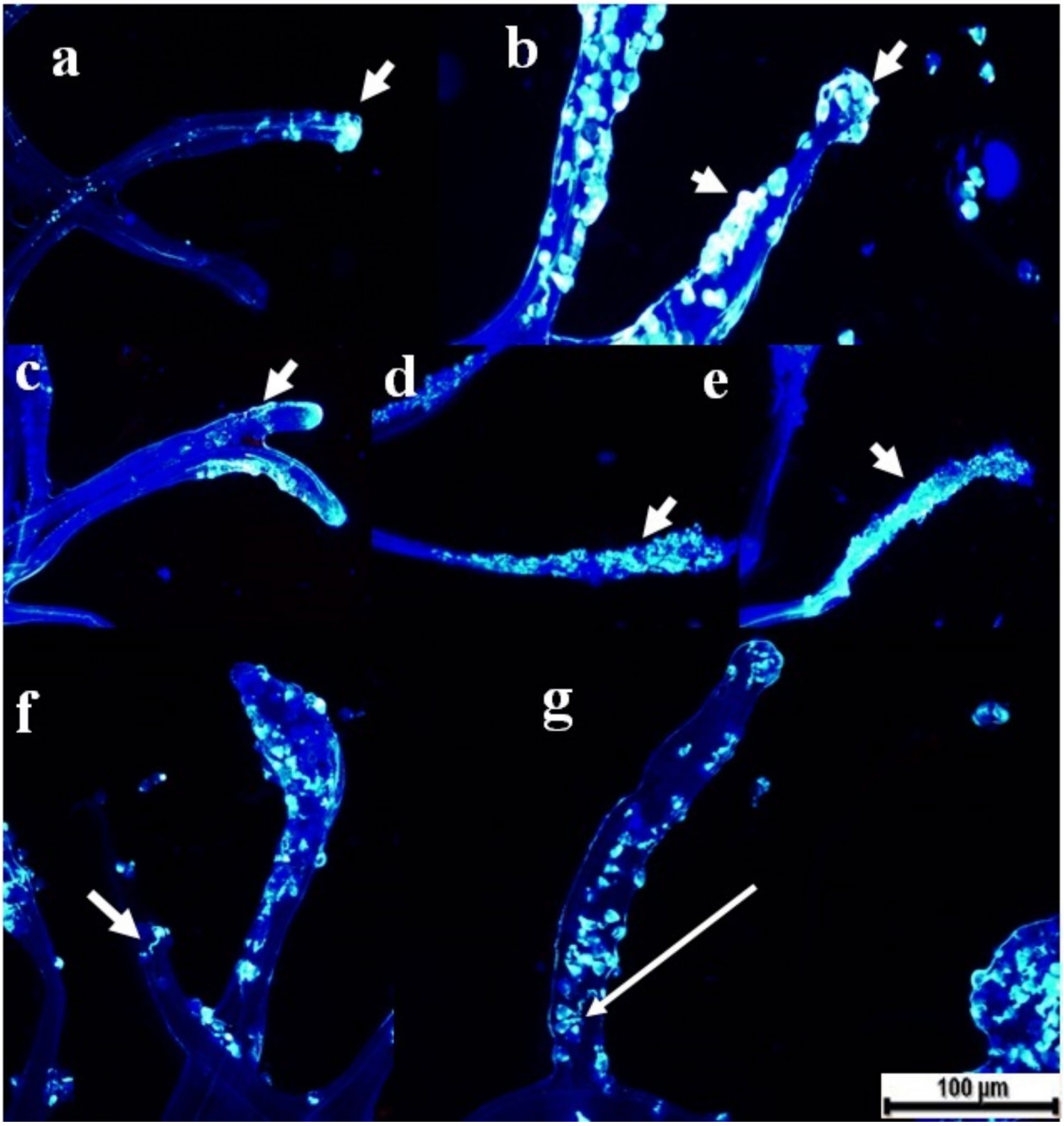


Fig. 5. Pollen germination in crosses with *L. grandiflorum* (**a**): Callose formation on the stigma surface (2 HAP) in *Lu* X *Lg* and, (**b**): Callose formation (2 HAP) *Lg* X *Lu* as indicated by the arrow mark pointing towards the aniline blue stained localized thickened callose deposition plugs visible under microscope; (**c**): Ungerminated pollen grains seen during longer hours after pollination in *Lu* X *Lg* (10 HAP) as indicated by the arrow mark pointing towards aniline blue stained small granular fluorescent areas; (**d**, **e**): Ungerminated pollen grains seen during longer hours after pollination in *Lg* X *Lu* (24 HAP) that start degradation/deformation; (**f**, **g**): Limited/restricted pollen grain germination on stigmatic surfaces in *Lg* X *Lu* (4 HAP) and *Lu* X *Lg* (6 HAP) wide crosses respectively.

hours of observations (Fig. 3c). The results indicated that the germinated pollen tubes were able to grow through different stylar regions at varying rates and successfully reached the ovule within 2 HAP. This is illustrated in Fig. 4b–d for *Lu*, Fig. 4f–h for *Lb*, and Fig. 4j–l for *Lg*, without encountering any visible obstacles in the growth path of the pollen tubes. The analysis revealed a noteworthy distinction in the PTG rate of both the wild relatives, *Lb* and *Lg*, when compared to the cultivated *Linum*, with a significance level of $p < 0.05$ (Table S3). This finding emphasizes the importance of studying these wild relatives for potential agricultural advancements.

Pollen tube growth in cross-pollinated pistils

In case of wide cross combination, with *Lu* X *Lb* and its reciprocal *Lb* X *Lu* it took only 2 HAP to reach to the stylar, ovary and ovular regions (Fig. 3d and e). In 2 HAP, data indicated that only 1.6% and 0.32% of PTs were able to penetrate the ovular region within the ovary in *Lu* X *Lb* and *Lb* X *Lu* respectively. However, the maximum percentage (2.82% and 1.25%) were demonstrated during 8 and 48 HAP in these cross combinations. The germinated PTs were observed growing with inconsistently variable rates in pistil without following any specific growth rate patterns. No significant differences were established ($p < 0.05$) in the PTG rate in the direct or reciprocal cross combination of cultivated *Linum* with its wild progenitor (*Lb*) (Table S3). With other distant cross combination i.e., *Lu* X *Lg* and its reciprocal *Lg* X *Lu*, again it took only 2 HAP to reach to the style and ovary regions without any PTG being visible in the ovular region in both of these crosses during this hour (Fig. 3f and g). The data indicated 0% pollen tubes penetrating the ovular region within the ovary in these crosse in first 2 HAP. However, the pollen tubes could be seen reaching up to the ovular region only in about 4 HAP in *Lu* X *Lg* (14.29%) and in *Lg* X *Lu* (9.03%). The maximum percentage (28% and 16.05%) were demonstrated during 24 and 8 HAP in these cross combinations. Again, in this case, the germinated PTs were observed growing with variable inconsistent rates in the observed pistils. Significant PTG rate differences ($p < 0.05$) were obtained in the pistil in crosses attempted with its wild relative *Lg*, that implies the occurrence of pre-fertilization barriers in the path of growing pollen tube (Table S3). The journey of pollen tube growth is represented in Fig. 4n–p for *Lu* X *Lb* and, Fig. 4r–t for its reciprocal *Lb* X *Lu*. For the crosses attempted with its wild relative *L. grandiflorum*, the pollen tube growth barriers started appearing right after the foreign pollen landed on the stigma surface of the other parent in the form of callose deposition (Fig. 5). The study of pollen-pistil interaction in the crosses *Lu* X *Lg* (illustrated with arrow marks in Fig. 6) and *Lg* X *Lu* (illustrated with arrow marks in Fig. 7) reveals significant abnormalities that cannot be overlooked. Observations included partially elongated, swollen, and twisted pollen tubes (PTs), as well as PT tips directed in the reverse orientation. Furthermore, we identified complex growth patterns alongside non-germinated pollen grains showing signs of degradation. These concerning behaviors of the growing PTs underscore the critical impact of delayed pollen tube arrival at the ovular region, highlighting an important area for further investigation in plant reproduction.

Discussion

Seed setting

Fertilization between diverse species within the same genus is expected to be successful only if there is some means of compatibility between both the parents. Once there happens a successful completion of a series on sequential events following pollination, as allowed by the coordinated gene and gene complexes of both pollen and pollen parents, successful fertilization takes place[37–39]. It is worth mentioning here that, capsules formation was observed during most of the attempts made under field conditions to an extent of about 70.12% in direct crosses and 21.97% in reciprocal cross combinations with *L. grandiflorum*(Fig. 1**).** However, our experimental results showed formation of non-viable chaffy seeds (Fig. S1) in cultivated *Linum* with its wild relative *Lg*(Table S1**).** Earlier studies also report unsuccessful attempts for obtaining interspecific hybrids between cultivated flax and *L. grandiflorum*[40] and attempts to get hybrids with embryo rescue have been also done in the past[41]. Since, capsules were formed without any viable seed development; we assume the occurrence of parthenocarpic development of fruits without affecting successful fertilization resulting into capsules with non-viable seeds. Wide hybridization as a driving force for the development of seedless or rudimentary fruits in higher plants have been discussed thoroughly[42,43]. Recently Badiger et al.[28], reported that formation of aborted embryo with partially filled seeds suffered from severe pre-zygotic barriers, because of interspecific hybridization in the crosses in *Abelmoschus* species.

Pollen vitality

On the other hand, pollen vitality is another important factor affecting the pollen germination percentage. Pollen represents a critical stage in the life cycle of plants, as viable pollen is crucial for efficient sexual plant reproduction. Reproductive fitness in wild plants is often determined by the quantity and quality of pollen grains produced. Both parameters can be considered as survival strategy in deciding reproductive fitness of natural populations growing spontaneously under resource limited marginal environments[44]. High pollen vitality in *L. bienne* (86.35%) and *L. grandiflorum* (91.30%) than the cultivated *L. usitatissimum* (69.92%) (Fig. 2) indicates towards the competitive ability of these wild species to capture more opportunities to affect efficient fertilization during sexual reproduction. Though, pollen vitality is an important factor for pollen germination and tube growth, these parameters were seemed to be drastically reduced (Table S2) in case of wide crosses with *L. grandiflorum*, despite appreciable pollen vitality percentage in *L. usitatissimum* (69.92%) and *L. grandiflorum*(91.30%) (Table 3). This emphasizes that high pollen vitality does not guarantee successful pollen germination and subsequent pollen tube growth inside the foreign style's transmitting tract when dealing with genetically distant parents. Pollen germination is a vital indicator of viability and provides valuable estimation for pollen tube growth. It is crucial to note that germination loss does not necessarily mean the pollen has died, but rather underscores the need for optimal germination conditions[45]. The genetic differences between cultivated and wild *L. grandiflorum* seems violating the congenial conditions required for optimal germination of pollen grains and subsequent pollen tube growth. Self-incompatibility arising from heterostyly is widely documented in various species linked to cultivated *Linum*, as mentioned previously. Mechanism of self-incompatibility (SI) between different floral morphs are also studied and the mechanism of SI among various floral forms in *Linum grandiflorum*[32,34], *Linum usitatissimum*[33] have been investigated in the past. Based on what we know, there is a lack of available literature on the germination and tube growth of foreign pollen grain on the stigma and style of a different species in flaxseed.

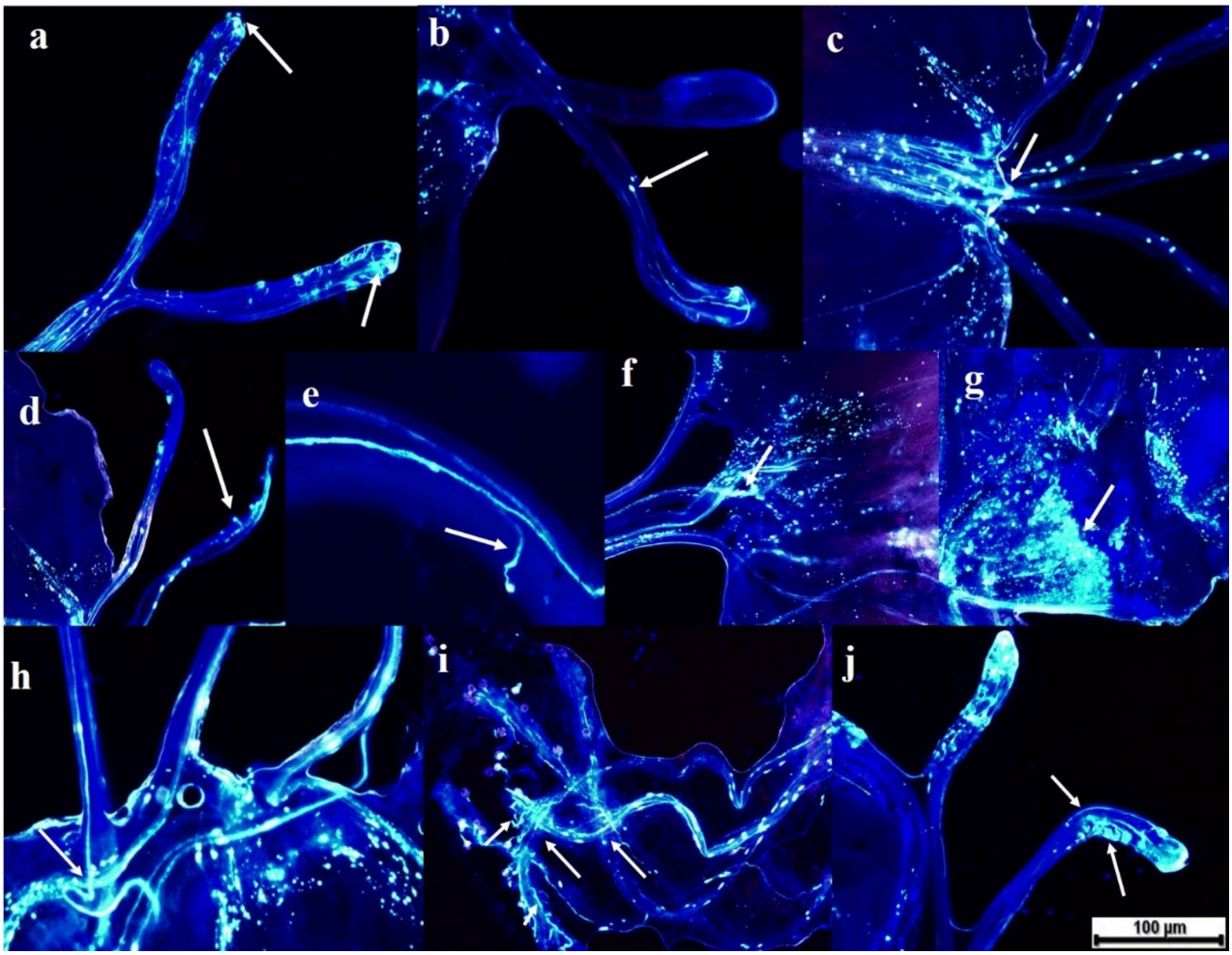


Fig. 6. Representation of activities taking place in *L. usitatissimum* during pollen-pistil interaction in wide crosses of *L. usitatissimum* X *L. grandiflorum*. (**a**): Partially elongated/restricted pollen tube growth due to callose deposition on the stigma in *Lu* X *Lg* (4 HAP); (**b**): Swollen pollen tube tip in mid stylar region (4 HAP in *Lu* X *Lg*); (**c**): Swollen pollen tube tip at the attachment region (4 HAP in *Lu* X *Lg*); (**d**, **e**): Twisted pollen tube growth in the upper and mid stylar region of pistil (4 HAP); (f): Swollen pollen tube tip at attachment region of ovary (4 HAP in *Lu* X *Lg*); (**g**): Inhibited pollen tubes at the attachment region of ovary (4 HAP in *Lu* X *Lg*); (**h**): Appearance of pollen tubes growing in reverse direction while reaching towards ovule (6 HAP); (i): Complex and convoluted pollen tube growth at middle stylar region (6 HAP); (**j**): Ungerminated pollen grains and terminated pollen tube growth (10 HAP).

Pollen germination on stigmatic surface

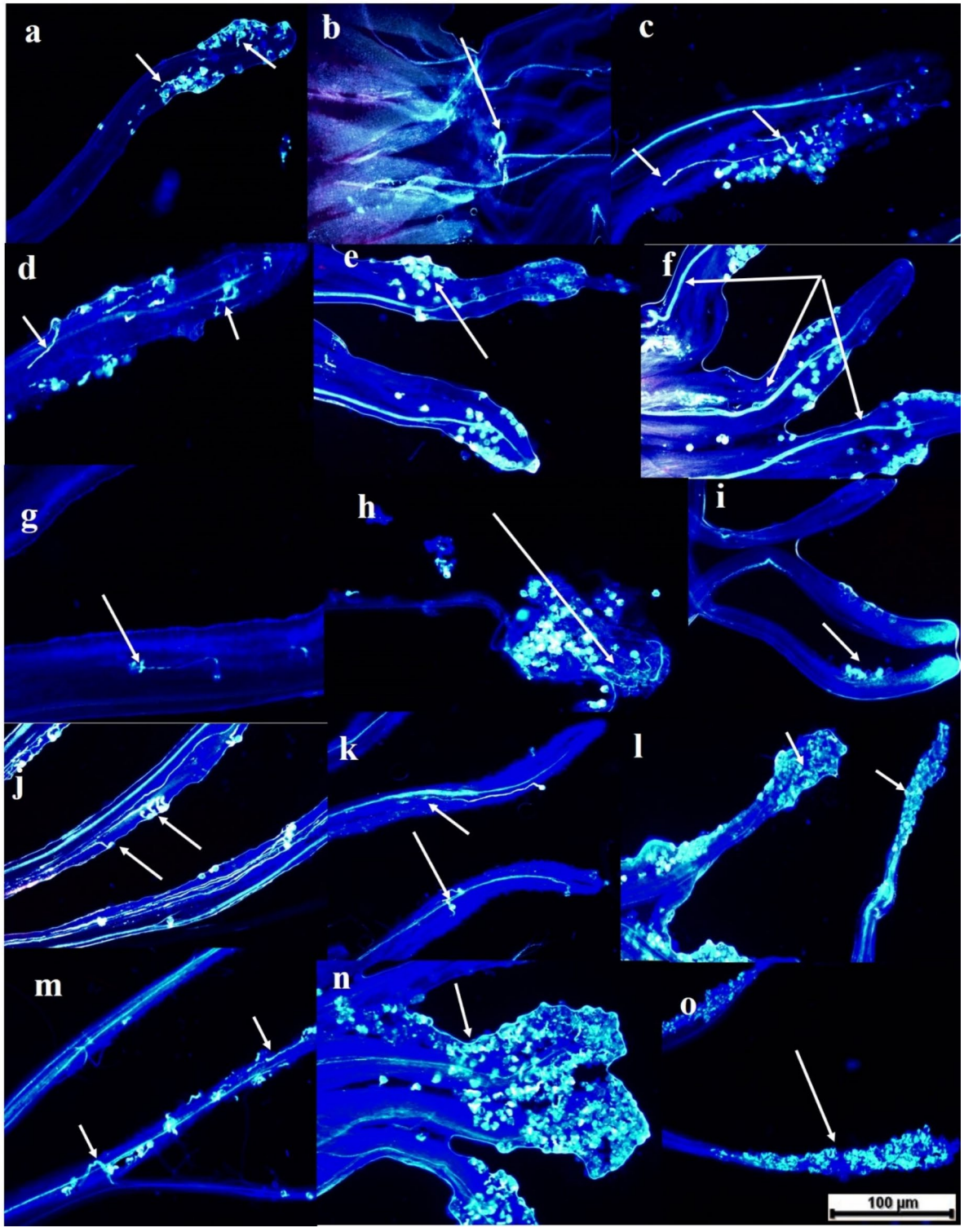
In case of self-pollination for the cultivated species *L. usitatissimum*, its wild progenitor *L. bienne* and, legitimate mating with its distant relative *L. grandiflorum*, the PG percentage were as high as 95% (Fig. 3a, b and c). Similarly, the PG percentage on the foreign stigma, when *L. bienne* was used either as pollen or pistil parent, were as good as in case of individual species when self-pollination was done (Fig. 3d and e). This can be accounted to the genetic similarity between the cultivated *Linum*species and its wild progenitor which is the immediate ancestor of modern cultivated flax[46]. However, when *L. grandiflorum* was used as pollen or pistil parent, the PG percentage were drastically reduced during different HAP (Fig. 3f and g). The high percentage of pollen germination on the stigma surface of the self-species was evident in the captured images too (Fig. 4a, e, i). The occurrence of high germination percentage may be explained by the hypothesis proposed by Lewis[47] that resulted into adaptation from allogamy to autogamy in flax following two phenomena. These may include either a mutation of activation of pollen specificities, the styles maintaining their incompatibility reaction or, the development of secondary fertility genes thwarting the incompatibility reaction or partial allogamy and the lack of complete auto-sterility. PG percentages and pollen tube (PT) lengths varied in flax styles for different crosses, including reciprocals and selfings, using cultivated *L. usitatissimum* species[33]. The maximum PG on the stigmatic surface of cultivated *Linum*, when *L. grandiflorum* served as pollen parent, could reach to a maximum of 10.92% only in 4 HAP. When used as pistil parent, the PG reached to a maximum of 10.33% only in 6 HAP. Varied response and reciprocal differences of pollen and pistil parents, on PG, PTG and compatibility parameters have been studied in wide hybridization of *Vigna* species[48]; pigeon pea[49], *Curcuma*[50]. Appreciable pollen germination was observed

withing 2 HAP in crosses involving *Lb* as distant parent (Fig. 4m and q). However, it was evident that there is a reproductive barrier imposed on the landed pollen grain during the pre-fertilization stage in crosses attempted with *Lg* as distant parent. Analysis of captured images under fluorescent microscope, indicated the formation of a thick callose layer on the stigma surface (Fig. 5a and b), where the callose deposition could be seen within the 2 HAP. In the incompatible cross combinations of *Lu* X *Lg*, and its reciprocal, larger proportions of pollen grains lying ungerminated on the foreign stigmatic surface could be observed even longer hours after (10 to 24 HAP) pollen landing (Fig. 5c, d and e) that started showing up some form of degradation. The main obstacles for PG included the callose layer forming within 2 HAP, which limited the germination of foreign pollen grains on stigmatic surfaces, taking 4 to 6 h (Fig. 5f and g). All flowering plants rely on the crucial process of sexual reproduction. This essential process is initiated when a pollen grain reaches the stigma of a pistil and gives rise to a vital pollen tube[51]. The journey to hybridization between different species within the same genus may encounter challenges, but groundbreaking researchers such as Dumas and Knox[52]and Chen and Kim[53] have championed the use of aniline blue to unravel the callose response, shedding light on the phenomena of rejection. Researchers have stressed the importance of utilizing aniline blue to track callose response, associated with pollen rejection. The Aniline Blue Fluorescent (ABF) method and fluorescence microscopy have been employed to extensively investigate PPI in a variety of crops such as sorghum[54], sesame[27]. However, studies for analyzing unsuccessful seed set because of pre-fertilization barrier due to incompatible PPI in *Linum*interspecific crosses is scarce. Callose is a crucial component of the cell wall, being dynamically deposited and degraded throughout pollen development[55]. In many angiosperms, a brief callose-rich cell wall forms around each microsporocyte in the anthers, while callose is also present on the outer pollen wall. Callose effectively separates microspores and acts as an impermeable barrier[56]. In species with callose-rich walls, mutants lacking callose production consistently produce inviable pollen[57]. Visible ungerminated pollen grains on the stigmatic surfaces in case of incompatible interspecific hybridization after longer hours of pollen landing may be explained due to deposition of the callose layer as an attempt to retain longer pollen viability (Fig. 5a–e). Callose provides a useful phenotypic bioassay in plant breeding to determine incompatibility system, pollen competition, stigma, and ovule viability and to understand the pathogen induced defense mechanism triggered in the plant systems[52,58]. The role of callose in pollen-stigma interactions has many analogies with host-parasite interactions as callose deposition is viewed as an indicator of biocommunication between pollen and stigma during PPI[52]. This pathogen-induced callose deposition functions as a chemical and physical defense mechanism for reinforcing plant cell wall and plays an essential role in the defense response to invading pathogens[58]. In *Linum*, it is assumed that callose deposition in response to non-self-pollen grains serves as a formidable barrier to pollen germination and pollen tube growth in incompatible distant crosses under the current study. The response of stigma callose is distinctly influenced by informational molecules from self or interspecific pollen grains. Dumas and Knox[52] proposed model establishes a compelling connection between callose, boron, and inhibitor synthesis akin to phytoalexins. In the model plant *Arabidopsis thaliana*two independent research groups identified twelve genes encoding putative callose synthase namely CalS (callose synthase) and GSL (glucan synthase-like) genes[59]. Callose wall plays an important role in normal pollen mother cell development. Recently, through genetic approach it is revealed that *OsGSL5* gene is responsible for callose deposition in anther locules and *OsGSL5*gene mutation resulted in anthers with less callose deposition, aberrant pollen mother cells and abnormal microspore[60]. Global transcriptome analysis showed that expression of *OsGSL5* was downregulated in the *Osspl* (*OsSPOROCYTELESS*) mutant which was defective in meiosis-specific callose deposition[61,62]. Similarly, we posit that a mutated or malfunctioning expression of genes responsible for synthesizing callose on the stigmatic surface when encountering non-self-pollen grains may present an opportunity to overcome pre-fertilization barriers arising from callose deposition. It is recommended to conduct further studies to explore the suppression of such genes and its potential impact on enabling the overcoming of callose-related barriers.

Pollen tube growth within the pistil

Irrespective of self- or cross-pollination, in all the studied three *Linum* species, and their wide cross combinations, the germinated pollen grains were able to grow and become visible within the various stylar regions of the pistil parent in different time intervals. Contradictory to the fact that the number of growing pollen tubes that would be expected to increase over time, our data showed an inconsistent growth rate in all the cases of self and cross pollination while growing towards the ovular region from stylar region (Fig. 3) compared to the pollen germination percentage. Just like self-pollinated pistils (Fig. 3a, b and c), wide crosses with *L. bienne* (Fig. 3d and e), were able to be seen growing PTs within various stylar regions in foreign pistils and seemed navigating ovular region within 2 HAP that indicates towards the compatibility of pollen pistil interaction in the wide crosses with its wild progenitor *L. bienne* where the foreign pollen tube is not discriminated as non-self. This non-discrimination might allow the PTs to be recognized as self-pollen-tube through various stylar regions. Though, no hurdles could be witnessed in the paths of growing PTs in case of self-pollinated pistils of *Lu* (Fig. 4b-d), *Lb* (Fig. 4f-h), and *Lg* (Fig. 4j-l) and compatible cross combinations with its wild progenitor (*Lb*) (Fig. 4n–p) and reciprocal (Fig. 4r–t), the rate of PTG varied over different time intervals to travel the path to reach up to the ovule. This trend underscores the need for further investigation into the factors influencing PT development over time.

However, the pollen grains that could successfully cross the germination barriers due to callose deposition on the stigmatic surface in *Lu* X *Lg* and its reciprocal, which were seen growing their PTs in the stylar regions of pistil parent within 2 HAP were unable to reach up to the ovular region of foreign pistil parent. Appearance of PTs growing in the ovular region for distant crosses with *L. grandiflorum* could be noticed only during 4 HAP irrespective of pistil parent (Fig. 3f and g). The delayed growth of PT in the foreign style was explained due to predominant resistance force due to a high intensity of PTs in the stigmatic surface[63]. Unlike self-pollinated



pistils and, crosses with its wild progenitor i.e., *L. bienne*, various kinds of aberrations were observed in the developing PTs, imposing barriers in the path of further growth in wide crosses with *L. grandiflorum*.

The major PTG inhibition occurred in the stylar region was, due to appearance of emerging deformed growth patterns in the developing tubes. These discordant growth patterns may be attributed to cause delayed arrival of PTs to ovular region of foreign pistil in wide crosses with *L. grandiflorum* (Fig. 3). According to Hodnett et al.[64], unfavorable conditions hindered the normal metabolism of the PT, leading to its malformation and subsequent reduction in growth towards the micropyle. Comprehensive analysis of PTG in the interspecific and intergeneric crosses as an indicator of pre-zygotic barrier is not just limited to field crops but have been extensively implied in horticultural crops too[65,66]. In stark contrast to the crosses attempted with *Lb*, where pollen tubes successfully navigated the ovular regions in both direct (Fig. 4m-p) and reciprocal (Fig. 4q–t) crosses, the distant crosses involving *Lg* revealed significant challenges. Here, the first signs of pollen tube growth inhibition were evident at

◀**Fig. 7**. Representation of activities taking place in *L. grandiflorum* during pollen-pistil interaction in wide crosses of *L. grandiflorum* X *L. usitatissimum*. (**a**): Restricted and twisted pollen tube growth (2 HAP); (**b**): Twisted pollen tube growth at attachment region of ovary (2 HAP); (**c**, **d**): Under-germinated pollen grains with terminated, twisted and swollen pollen tube (4 HAP); (**e**): Ungerminated pollen grains are observed even after 4 HAP; (**f**): Reduced numbers of pollen tubes growing in the stylar region along with non-germinated pollen grains (4 HAP); (**g**): Ruptured/bifurcated pollen tube tip in the stylar region (4 HAP); (**h**): Convoluted pollen tube growth in the mid-stylar region (10 HAP); (**i**): Pollen grains are seen ungerminated on foreign stigma of *L. grandiflorum* (10 HAP); (**j**): Considerable pollen tube growth is visible only in 6 HAP with elongated pollen tube growth in the stylar region. Some pollen tubes can still be observed with partial growth at this stage; (**k**): Delayed pollen tube growth with swollen tip (6 HAP).; (**l**): Considerably non germinated pollen grains observed on stigmatic surface (8 HAP); (**m**): Many pollen grains germinated with swollen pollen tube tips and twisted growth with random growth patterns can be seen in the stylar region even 10 HAP; (**n**): Complex, random pollen tube growth pattern (10 HAP); (**o**): Non-germinated pollen grains start degradation (24 HAP).

the stigma surface, primarily due to excessive callose deposition (Fig. 5). This initial barrier was soon followed by the emergence of various abnormalities for the growing PTs in the pistils of the foreign parent, further complicating the fertilization process (Figs. 6 and 7).

Most of the nonconductive circumstances started appearing during the 2–4 HAP (Fig. 6a–g). Partially elongated PTs along with the non-germinated pollen grains could still be seen in the upper stylar regions of *L. usitatissimum* during this time duration (Fig. 6a). Molecular aspects better explain the reasons behind the rejection of incompatible PTG. Some studies mention role of putative protein O-fucosyltransferase facilitating PT penetration through the stigma-style interface[67]. Stylar region comprises various chemical signals that reside in the extracellular matrix of transmitting tract play crucial roles in the recognition of self or non-self-PT. There are various substances existing in the extracellular matrix of the transmitting tract that reject non-self-PTs and the loss-of-function of the pistil-side barrier will result in self-incompatibility transition to self-compatibility[68]. Various biomolecules involved in guiding the PT through the transmitting tract have been extensively elaborated and reviewed by Zheng et al.[69] and recently by Cheung et al.[38].

Swelling of PTs at the tip was traced through the florescent image analysis in the mid-stylar (Fig. 6b) and attachment regions of the ovary (Fig. 6c) in the pistil. Non-penetration of the ovular region even at 96 HAP was reported in the studies of Gong et al.[70]; where they reported that PT barely entered the ovule, but remained at the base of the style and became swollen in another oilseed plant species *Camellia oleifera*. The control of PTG by protein factors was addressed by Mei et al.[71] in model plant *Arabidopsis thaliana*. Twisted PTG was witnessed in the upper (Fig. 6d) and mid-stylar (Fig. 6e) regions along with severe inhibition of the growing PT in the ovary (Fig. 6f and g). The appearance of PTs growing in reverse direction (Fig. 6h) while reaching towards the ovule, complex and convoluted PTG at the mid-stylar region (Fig. 6i) were noticed during 6 HAP. Partially germinated pollen grains with terminated PTG (Fig. 6j) were still visible even after longer hours of pollination (10 HAP). Thus, the initial 4 h were proved critical and crucial from the PG and PTG point of view. In the interspecific crosses, there were frequent observations of delayed growth of PTs, as well as other structural abnormalities such as twisting, swelling, high branching, a bi-furcated tip, PTG before reaching the ovary and inability to navigate to micropyle and variations in callose form. These variations included reverse orientation and irregularity in callose plugs along the PT. Such abnormalities attributed for delayed pollination and delayed pollen tube growth resulting into incompatible interaction[72].

In case of distant crosses made with *L. grandiflorum* as pistil parent, almost similar abnormality patterns were observed during PTG of *L. usitatissimum*. Within 2 HAP, the pre-fertilization barriers started appearing in the form of restricted and twisted PTG in the upper stylar region (Fig. 7a). As soon as the PT arrives at the attachment region in of ovary at the lower stylar region, some PT faced twisted growth patterns (Fig. 7b). Under-germinated pollen grains with terminated, twisted and, swollen PT was most frequent during the 4 HAP (Fig. 7c and d). Non-germinated pollen grains were quite obviously visible during different HAP from 2 to 24 HAP (Fig. 7e). Large proportions of ungerminated pollen grains along with consistently growing PTs were noticed in the stylar region (Fig. 7f). Reduced number of PTs passing through ovular regions affecting fertilization after longer hours of pollination taking up to 18 to 24 h have been reported in wide crosses of *Vigna* spp.[73]). Ruptured and bifurcated PT tip in the stylar region could also be witnessed as soon as 4 h after pollen landed and germinated on foreign pistil (Fig. 7g). Convoluted PTG (Fig. 7h), partial or delayed PTG (Fig. 7i) with swollen tip (Fig. 7j) were visible even after longer durations following pollination. Pollen tube growth termination at mid stylar region with swollen tip 6 HAP *Lg* X *Lu* delayed pollen germination could be observed even after 6 HAP (Fig. 7k and l). Many pollen grains germinated with swollen PT tips and twisted growth patterns showing random growth patterns could be seen in the stylar region even 10–12 HAP (Fig. 7m and n).

The pre-fertilization barriers were predominant in wide crosses with *L. grandiflorum*, that operated in all the stages resulting into delayed PTG. These barriers not only acted on the stigmatic surfaces but also had operated gradually and mildly at various growth stages. No PTs could be noticed penetrating ovular region in wide crosses with *L. grandiflorum* till 4 HAP as opposed in crosses attempted with *L. bienne*, where the ovular penetration occurred within 2 HAP (Figs. 3 and 4m–t**).** Low levels of PT penetration and PTG in the wide crosses due to operative pre-fertilization barriers have been advocated in *Vigna* spp[73]., in Sesame spp[74]., in *Abelmoschus* species[28]. Based on image analysis, the non-arrival of the PT within the first four HAP could be attributed to the various kinds of pre-fertilization barriers and deformities appearing during PTG. The first two to four hours seemed critical for the PT growth in wide crosses with *L. grandiflorum*. Though, the PT was able to reach up to

the attachment region of ovary at the lower stylar region of pistil within the first 2 HAP, they remained unable to penetrate the ovular region until 4 HAP.

Interspecific hybridization in *Linum* has been advocated in the past and has been successfully used in both West European and East European cultivars. For example, *Linum crepitans* L. was employed to introduce early maturity traits into the *L. usitatissimum*L. However, the potential of these early maturing types was not fully realized in subsequent breeding efforts. While interspecies crossing generally exhibits low effectiveness, this can be improved through the application of embryo tissue culture and the cultivation of immature isolated embryos under in vitro conditions[75,76]. In situations where natural interspecific hybridization in *Linum*fails due to pollen-stigma incompatibility, the utilization of advanced techniques such as protoplast fusion and embryo rescue holds immense promise for obtaining successful interspecific hybrids. The ongoing research on protoplast culture and isolated immature embryos of distant hybridization of flax will undoubtedly yield valuable insights into the selection of optimal medium and culture conditions, paving the way for groundbreaking advancements in this field[76]. In Wang's[77] study, interspecific hybridization between *L. usitatissimum* and *L. perenne* was conducted. The results demonstrated that repeat-pollination alongside the use of plant growth regulators (such as GA_3, NAA, and 2,4-D) proved to be an effective method for overcoming interspecific cross-incompatibility in flax. Production of haploids followed by genotype stabilization in the case of interspecific hybridization for extension of genetic variability in *Linum* is one of the unconventional methods for producing interspecific hybrids. For this purpose, the application of biochemical methods has been studied and suggested.

Conclusion

The present study revealed that the interaction between the pollen and pistil is inhibited in the distant crosses among *Lu* X *Lg* due to both temporal and spatial pre-fertilization barriers. Callose deposition at the stigmatic surface of foreign pistils within 2 HAP leading to inability of pollen grains to penetrate the stylar tissue appeared as the major barrier. Various kinds of aberrations started appearing during the 2–4 HAP, in the developing PTs imposing barriers in further growth. Delayed PTG was observed mainly due to the discordant conditions appearing in the various stylar regions. The complexity of interspecific hybridization was observed in terms of arrest of pollen tube (PT) growth in the ovary, ruptured, twisted and swollen pollen tube tip, tube growth in reverse direction, convoluted and terminated growth patterns. Although interspecies crossover is often less efficient, it can be made more effective by using embryo rescue and cultivating isolated, immature embryos in vitro. We propose that the disruption of genes responsible for producing callose on the stigmatic surface when encountering non-self-pollen grains could offer a promising opportunity to overcome pre-fertilization barriers associated with callose deposition. Further studies are recommended to investigate the suppression of these genes and the potential impact on surmounting callose-related barriers. When pollen-stigma incompatibility prevents natural interspecific hybridization in *Linum* from occurring, the application of cutting-edge methods like protoplast fusion and embryo rescue presents great potential for producing interspecific hybrids that succeed. The results show that while distant hybridization with *L. grandiflorum* is less efficient, pollen tubes can still navigate the ovular tissues, albeit with some delay. This finding opens avenues for investigating factors that hinder viable seed formation, enhancing our understanding of reproductive success in distant hybridization with this species.

Data availability

No datasets were generated or analysed during the current study.

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V.K.R: Experimentation and writing—original draft, Data collection; A.Y. & M.R.: Supervision; V.K. & D.W.: Investigation, review, and editing; P.P.: Experimentation; M.S.: Conceptualization, supervision, writing—original draft; funding acquisition; G.P.S.: funding acquisition; project administration.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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