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Nuclear-cytoplasmic interaction for stigma receptivity in Indian mustard (*Brassica juncea*) hybrid development

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ABSTRACT

Field experiments were conducted during 2020–21 and 2021–22 at the research farm of ICAR-Indian Agricultural Research Institute, New Delhi to study the response of stigma receptivity in 30 new CMS lines, based on 3 new cytoplasmic male sterile (CMS) sources namely, M*oricandia, erucoides* and *berthautii* of Indian mustard [*Brassica juncea* (L.) Czern.] using two traits, i.e. per cent siliquae set and seed set per siliquae which are important for hybrid seed yield in seed production. CMS lines showed varying responses to distinct genetic backgrounds. Per cent siliquae set and seed set per siliquae changed due to cytoplasmic-nuclear interaction. Studies on stigma receptivity evaluation using manual pollination up to 7 days since anthesis revealed that it varies among the 3 CMS sources of *Brassica juncea*. Peak stigma receptivity reached up to 2 to 3 days following flower opening. Among the 3 CMS sources *berthautii* showed higher stigma receptivity compared to *Moricandia* and *erucoides*. Among the nuclear genotypes, Pusa Tarak was the most promising. The new CMS sources would be useful for hybrid development based on higher stigma receptivity for hybrid seed production. These findings will aid in the selection of appropriate nucleo-cytoplasmic combinations for use in the *B. juncea* hybrid breeding programmes.

Keywords: Artificial pollination, *Brassica juncea*, Cytoplasmic male sterile line, Stigma receptivity

Mustard accounts for one-third of the country's oil production after soybean. India holds 19.29 and 11.27% of global mustard acreage and production, respectively (USDA 2013). However, there is a huge gap between demand and production. Mustard seed growers tend to use lower quality seed in conventional production systems and opt for higher quality seed (foundation or certified seed) for seed production purposes (Layek *et al.* 2021). Indian mustard [*Brassica juncea* (L.) Czern.] is a member of the Cruciferae (Brassicaceae) family and is an important oilseed crop grown in winter (*rabi*) season in India. Mustard flowers are a rich source of protein (pollen) and sugar (nectar), making them a popular site for pollinating insects primarily honey bees. Although Indian mustard is a naturally autogamous plant, out-crossing occurs frequently in the crop, ranging from 5 to 30% depending on environmental conditions and the diversity of pollinating insects (Kumar *et al.* 2013).

A significant development in mustard improvement has been witnessed in recent years with the introduction of hybrid. Hybrid mustard, developed through crossbreeding, offers farmers improved yield potential, enhanced resistance, and superior oil quality. Among the notable hybrid varieties, NRCHB-506 has emerged as a game-changer, paving the way for a new era of mustard production in India. In *B. juncea*, a number of cytoplasmic male sterility (CMS) systems have been created (Rawat and Anand 1979).

The future of hybrid mustard holds great promise, especially in the context of cytoplasmic male sterility (CMS) system (Panjabi *et al.* 2019). New CMS and fertility restoration systems have been developed using the cytoplasm of wild species *Moricandia arvensis* (Prakash *et al.* 2001), *Diplotaxis erucoides* (Bhat *et al.* 2006) and *Diplotaxis berthautii* (Bhat *et al.* 2008). CMS plants with high stigma receptivity facilitate efficient pollination and fertilization, leading to increased seed set and improved hybrid seed yield. Hybrid mustard, utilizing CMS line, ensures high genetic purity in the resulting hybrid seeds. Hence an experiment was planed to study the stigma receptivity period in terms of seed setting percentage and number of seeds per siliquae in the 30 new CMS lines of Indan mustard for selection of parental lines for hybrid development.

MATERIALS AND METHODS

Plant material: The experimental material consisted of

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10 diverse *B. juncea* maintainer (B) lines and 3 CMS (A) lines carrying 3 different sterility-inducing cytoplasms, viz. *Diplotaxis berthautii* (*ber*), *Diplotaxis erucoides* (*eru*) and *Moricandia arvensis* (*mori*) in each of the 10 maintainers (nuclear) backgrounds (Table 1). The CMS lines and their respective maintainers were sown in 4 row plots of 5 m row length keeping 45 cm row to row and 15 cm plant to plant distance during winter (*rabi*) seasons of 2020–21 and 2021–22 at the research farm of ICAR-Indian Agricultural Research Institute, New Delhi. All recommended agronomic practices were followed to raise the crop.

Seven randomly selected plants of each CMS line were identified and tagged for hand pollination. The experiment was conducted on 10–15 inflorescences with 3–5 flowers in each inflorescence of the CMS lines in peak flowering period. Flower buds in an inflorescence likely to open on the next day were covered with a butter paper bag. All open flowers and immature buds were removed while bagging the inflorescences. On the following day, freshly opened flowers were allowed in each inflorescence. Thus, one plant was fixed for pollination on each day. Pollens from freshly opened flowers of the respective maintainer line were used for pollination each day. Pollination was done up to 7 days in 2020–21 and 2021–22. Inflorescences were covered again after pollination.

Data were recorded on number of per cent siliquae set and seeds per siliquae on the pollinated flowers of each CMS line. The data recorded in the two years were not significantly different, hence we pooled the data for statistical analysis. Stigma receptivity was determined using the siliquae set percentage and number of seeds per siliquae formed, following hand pollination up to a period of 7 days since flowering. The per cent siliquae set was calculated as:

Per cent siliquae set = Average no. of siliquae with seed formed per plant/Average no. of flowers pollinated per plant × 100

Number of seed set per siliquae = Average no. of seed set/Average no. of siliquae

RESULTS AND DISCUSSION

Brassica juncea inflorescence is an elongated corymbose raceme that grows terminally on the main stem and branches and bears vivid yellow flowers (Pua and Dogulas 2004). In hybrid mustard production, CMS lines are used as female (A line) parents, and fertile line possessing restorer gene serve as the male (R line) parents. The resulting hybrid seeds exhibit desirable traits and high yield potential. The use of CMS lines in hybrid mustard production enhances the efficiency of seed production as it eliminates the need for manual emasculation (removal of male reproductive organs) in female parent. This simplifies the hybridization process, reduces labour costs, and increases the scale of hybrid seed production (Ramanathan 2004). Further genetic purity contributes to the uniformity and stability of hybrid mustard crops, resulting in consistent yield performance and quality. Furthermore, the combination of CMS line with fertile line promotes hybrid vigour, which manifests as increased vigour, yield, and overall performance of the hybrid offspring (Ramanathan 2004).

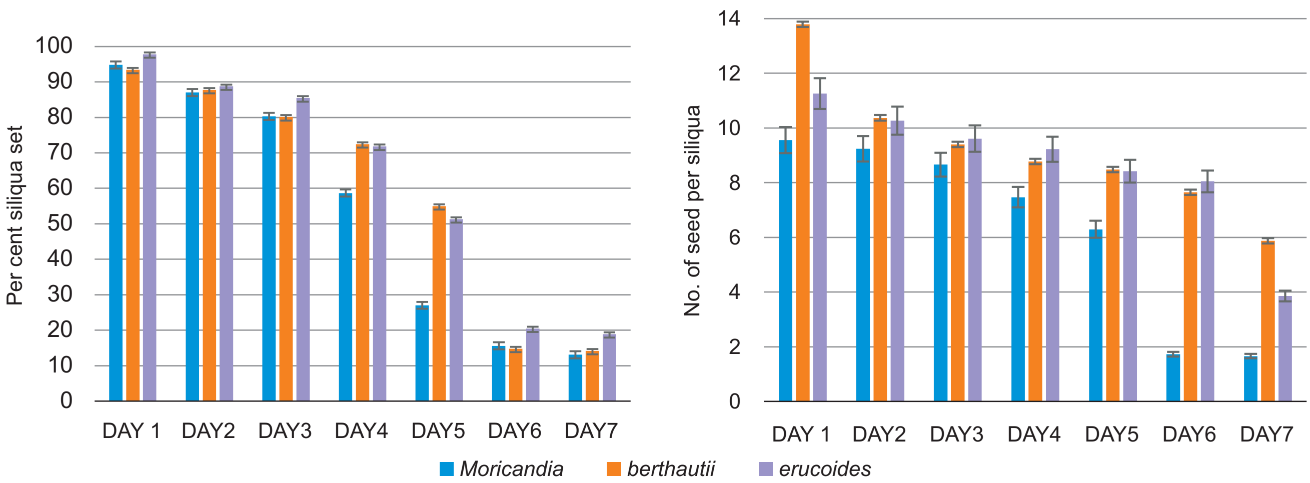
Reproductive ability of a variety and/or genetic stock depends on its floral biology, pollinating system and prevailing environmental conditions. Stigma receptivity of flower is an important attribute defining reproductive potential of a genotype. Genetic factors of the plant(s), pollinators and environmental factors determine reproductive system in Indian mustard.

Stigma receptivity duration in CMS sources: In our study a reduction of per cent siliqua setting and development up to 7 days from flower opening was recorded in all the CMS lines. The number of siliquae with seed declined with an increase in flower age at pollination time. All the CMS lines showed more than 50% siliquae set up to 4 days after flower opening. Similarly, the CMS lines had more than 6 seeds per siliquae up to 4 days of flower opening. However, the CMS lines did not show complete loss of stigma receptivity even by 7 days of flower opening as indicated by siliquae set percentage and number of seeds per siliquae. The CMS source *D. berthautii* had 5–6 seeds per siliqua when flowers pollinated even on 7th day after flower opening. The peak stigma receptivity in these three CMS lines was in freshly opened flower (i.e., on the first day of pollination) (Fig 1).

Studies have shown that stigma receptivity is genetically controlled and varies among different varieties of Indian mustard. For example, some varieties have a longer period of stigma receptivity, which increases the chance of success of fertilization (Banga and Banga 2017). Environmental factors, such as temperature and humidity, can influence stigma receptivity in Indian mustard. High temperature and low humidity can cause the stigma to dry out and become less receptive to pollen. The previous studies show that the variation observed in pod setting at different stages could be attributed to the inherent developmental changes in stigma and embryo sac of the female flowers. Mankar *et al.* (2007) observed a similar trend for stigma receptivity in *B. juncea*, reporting that the stigma may remain receptive 6–8 days after anthesis though the degree of receptivity reduced drastically (60%) after 3 days. Rai (1991) reported that stigma in *Brassica* remains receptive for 6 days. Maximum stigma receptivity in *B. juncea* was reported to be one day before the opening of flower (Labana and Banga 1984).

Table 1 Maintainer and new CMS lines used for the study

Maintainer line	NPJ 139	Pusa Kisan	Laxmi	SEJ 8	Pusa Agrani	NPJ 112	Pusa Tarak	Pusa Mustard 30	NPJ 161	NPJ 93
New CMS source	*Moricandia arvensis*	*Diplotaxis erucoides*	*Diplotaxis berthautii*							



CD (P=0.05): DAYS, 0.858; CMS, 1.311; DAYS × CMS, 2.271

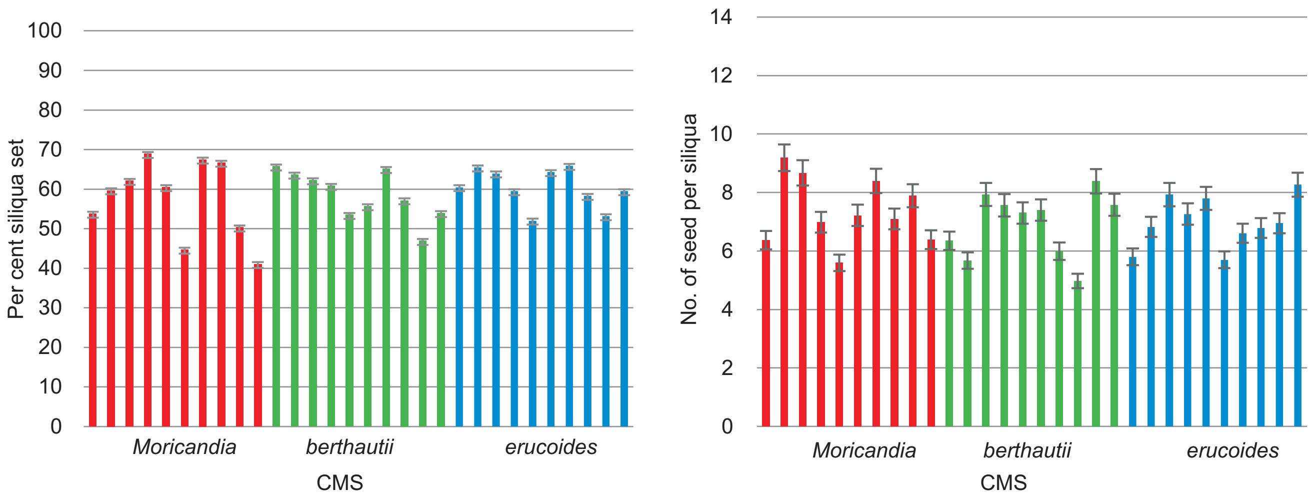
CD (P=0.05): DAYS, 0.117; CMS, 0.179; DAYS × CMS, 0.310

Fig 1 Variation in per cent siliquae set and no. of seed per siliquae in 3 CMS, bases (consisting of 10 CMS lines in each) up to 7 days (day 1 to day 7) of pollination after flower opening.

In *Brassica* spp. CMS lines with *Moricandia* cytoplasm, recorded the stigma receptivity up to 6 days after anthesis (Chakrabarty *et al.* 2007). In another study, stigma of CMS lines of *B. juncea* was receptive for 6–8 days after anthesis (Mankar *et al.* 2007). In protogynous lines of *B. juncea*, the duration of maximum stigma receptivity was reported to be up to 3 days after stigma protrusion (Chakrabarty *et al.* 2011), though the seed set was observed up to 10 days after stigma protrusion.

Stigma receptivity in CMS sources and all nuclear backgrounds: Per cent seed set and number of seeds/siliquae under manual pollination ranged from 41–68% and 4–9 seeds per siliquae among the CMS lines in a period of 7 days after flower opening. In case of *Moricandia* based CMS lines mean of per cent siliquae set ranged from 5–9 seeds per siliqua. Similarly, in case of *berthautii*, mean of per cent siliqua set ranged from 46–66% and 4–9 seed per siliqua. In case of *erucoides* mean of per cent siliqua set ranged from 52–66% and 5–9 seed per siliquae (Fig 2). The CMS lines possessing longer duration of stigma receptivity are useful for higher seed set and hybrid seed production. There is a need of integrating seed set data from different days of pollination either by manual controlled pollination or by open pollination in hybrid seed production plot to select parental line combination suitable for hybrid development.

Besides plant, pollinator and environmental factors, stigma receptivity is also regulated by several plant molecular and biochemical mechanisms, such as the expression of genes encoding stigma-specific proteins synthesis and secretion of signalling molecules, and the activity of enzymes involved in the metabolism of carbohydrates, lipids, and proteins. For example, the expression of genes encoding extension and arabinogalactan proteins has been shown to be associated with stigma receptivity in Arabidopsis, tobacco,



CD (P=0.05): DAYS, 0.644; CMS, 0.331; DAYS × CMS: 0.703

CD (P=0.05): DAYS, 0.095; CMS, 0.079; DAYS × CMS: 0.250

Fig 2 Variation in mean per cent siliquae set and seeds per siliquae in 3 CMS, bases (each in 10 nuclear backgrounds) over a period of 7 days of pollination since flower opening.

and petunia (Qin and Li 2020). A successful hybrid seed production can result in a higher yield and better quality of hybrid seeds, which can increase the profitability of farmers. In addition, successful hybrid seed production can reduce the cost of seed production.

Stigma receptivity in different CMS sources and in nuclear backgrounds: The three CMS bases each in 10 different nuclear/varietal backgrounds showed significantly different siliquae set percentage and number of seeds per siliquae. Among the *Moricandia* based CMS lines, SEJ 8, Pusa Mustard 30 and Pusa Tarak showed an average of 68% (ranging 19–91), 66% (ranging 13–98) and 67% (ranging 24–94) siliqua set, and 7 (ranging 2–9), 7 (ranging 3–9) and 8 ranging (4–10) seeds per siliqua, respectively (Fig 3).

Similarly, in case of *berthautii* CMS base, Pusa Tarak, Pusa Kisan and NPJ 139 showed 71–72%, 68–69% and 61–62% siliqua set and 5–6, 4–5 and 5–6 seed per siliqua, respectively (Fig 3). In case of *erucoides* CMS base, NPJ 112, Pusa Tarak and Laxmi showed 66–67%, 67–68% and 61–62% siliqua set and 4–5, 6–7 and 7–8 seed per siliqua, respectively (Fig 3).

Stigma pass through different functional stages during their development from being immature, receptive, and degenerated (Heslop-Harrison 2000). In some species, stigmas can collect pollen before the physiological receptivity (i.e. pollen can germinate and grow in the pistil), for example, by opening the stigmatic lobes and papillae (stigma flaring) or by producing stigmatic secretions (Sanzol *et al.* 2003, Yi *et al.* 2006). Delayed/extended stigma receptivity period is desirable in case of male sterile system being used as female line in hybrid seed production and between outcross pollen from different donors. Moreover, changes in paternal diversity also seem to affect the selective advantage of delayed stigma receptivity, which was the most important contribution of the present study. The period during which the stigma remains receptive is a critical factor in achieving successful pollination of a male sterile line, as no pollen is available in the flower of the female parent to pollinate itself. Studies have shown that male sterile female lines exhibit an increased duration of stigma receptivity, likely due to the inherent tendency of pollen-free flowers to remain receptive for longer periods in order to be pollinated by foreign pollens. Optimum sowing dates have resulted in stigmas remaining receptive for 5–6 days after opening, with the highest receptivity occurring on the day of anthesis. Following this, a gradual decrease in stigma receptivity is observed in terms of siliqua and seed setting, indicating that the age of the flower plays a role in determining its receptivity (Maity *et al.* 2019).

In *Brassica* spp. heterosis is a well-known phenomenon. Search for development of new diverse base in CMS system in cultivated crop species for hybrid development has been a researchable area. The future of hybrid mustard depends on broadening the genetic diversity of the A line pool. The success of a hybrid depends on higher rate of seed production. By incorporating diverse CMS sources in female line development with varying stigma receptivity, breeders can introduce new and desirable traits into hybrid varieties. One way to increase the success of hybrid seed production is to ensure that the female parent plants are at the optimal stage of stigma receptivity when pollination is carried out. Delayed stigma receptivity enhances outcrossing, and increases competition among pollen grains. Hybrid seed production depends on male sterility system's stability, availability of adequate pollinator population in a region, plant/flower factors including stigma receptivity and pollen fertility and its abundance in parental lines. Easy seed production methods a prerequisite for achieving a success in hybrid seed production method popularization among the famers. According to Sanzol and Herrero (2001), three major processes namely, stigma receptivity, pollen tube dynamics and ovule lifespan during the reproductive process limits the effective pollination time (EPP). Success of reproductive or artificial pollination depends on the timing and length of the stigma's receptivity. The seed set method is the most reliable for stigma receptivity, followed by artificial pollination.

In Arabidopsis, the maintenance of stigma receptivity to accept compatible pollen relies on a highly redundant mitogen-activated protein kinase (MAPK) cascade. Genetic investigations reveal that five MAPK kinases (MKK1/2/3/7/9) in the stigma play a crucial role in transmitting upstream signals to two MPKs (MPK3/4) responsible for facilitating compatible pollination. When the functions of these five MKKs are compromised in a quintuple mutant (mkk1/2/3RNAi/mkk7/9), pollination defects similar to those observed in the mpk4RNAi/mpk3 double mutant are manifested. Additionally, it has been discovered that this MAPK nexus ultimately converges on Exo70A1, a previously identified factor essential for stigma receptivity during pollination (Jamshed *et al.* 2020).

The results of the study comprising of 30 new CMS lines for two years can be summarised by reporting that stigma receptivity in the A (CMS) line plays a critical role in successful hybridization, genetic purity, and the incorporation of desirable traits. Through the selection and development of A lines with optimal stigma receptivity, breeders can drive the advancement of hybrid mustard, ensuring its relevance in meeting the challenges and demands of sustainable agriculture.The results on stigma receptivity duration ranged up to 7 days in the three new CMS bases in 10 nuclear backgrounds each. The superior CMS and restores combination in *Moricandia* were SEJ 8, PM 30 and Pusa Tarak; in *berthautii* Pusa Tarak, Pusa Kisan and NPJ 139; and in *erucoides* NPJ112, Laxmi and Pusa Tarak. It is suggested to use these CMS lines for superior hybrid seed production using a heterotic restorer line as a pollen parent. Among the 3 CMS sources *berthautii* showed higher stigma receptivity compared to *Moricandia* and *erucoides*. Among the nuclear genotypes Pusa Tarak was found most promising with all the three CMS sources based on higher stigma receptivity for hybrid seed production. The future of hybrid mustard, particularly in relation to cytoplasmic male sterile lines, is promising. The utilization of new CMS lines in hybrid mustard production improves seed production

Fig 3 Variation in per cent siliqua set and seed per siliqua in 3 CMS in each maintainer background up to 7 days of pollination from flower opening.

efficiency, ensures genetic purity, harnesses hybrid vigour, and allows for trait stacking and genetic improvement.

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