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Low temperature-induced aberrations in male and female reproductive organ development cause flower abortion in chickpea

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Abstract

Chickpea (*Cicer arietinum* L.) is susceptible to low temperature (LT) at reproductive stage. LT causes flower abortion and delays pod set in chickpea until terminal drought becomes an issue, thereby decreasing yield potential. In chickpea, flower and anther/pollen development as well as LT-induced abnormalities on anther and pollen development are described inadequately. In the present manuscript, we report flower development stages, anther development stages, and aberrations in male gamete formation in chickpea under LT. Flower length was linearly correlated to flower and anther stages and can be used to predict these stages in chickpea. LT affected male gamete development in a flower/anther age-dependent manner where outcome ranged from no pollen formation to pollen sterility or no anther dehiscence to delayed dehiscence. In anthers, LT inhibited microsporogenesis, microgametogenesis, tapetum degeneration, breakage of septum and stomium, and induced pollen sterility. Whereas disruption of male function was the prime cause of abortion in flowers below vacuolated pollen stage, flower abortion was due to a combination of male and female reproductive functions in flowers with mature pollen. The study will help in elucidating mechanisms governing flower development, anther and pollen development, and tolerance/susceptibility to LT.

KEYWORDS

anther dehiscence, anther development stages, female reproductive traits, flower development stages, male gametogenesis, microgametogenesis, microsporogenesis, ovule viability, stigma receptivity

1 | INTRODUCTION

Chickpea (*Cicer arietinum* L.) is the third most important edible legume in the world (Croser, Clarke, Siddique, & Khan, 2003). Being native of the Mediterranean region, chickpea is susceptible to cold at reproductive stage, delaying effectively the flowering as well as podding until drought sets in that further reduces the yields. Low temperatures (LT) at flowering stage in chickpea cause structural and functional abnormalities in reproductive organs, leading to failure of fertilization or premature abortion of flowers and pods (Srinivasan, Johansen, & Saxena, 1998; Croser et al., 2003; Clarke & Siddique, 2004; Nayyar, Bains, & Kumar, 2005,Nayyar, Chander, Kumar, & Bains, 2005; Kumar, Nayyar, Bhanwara, & Upadhyaya, 2010). LT in chickpea also induces pollen sterility, pollen tube distortion, ovule abortion, and reduces fruit set (Kumar et al., 2011; Srinivasan, Saxena, & Johansen, 1999; Thakur, Kumar, Malik, Berger, & Nayyar, 2010). Induction of pollen sterility under LT is considered to be the major reason for flower abortion in chickpea (Kumar et al., 2010); however, mechanisms underlying

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LT-induced pollen sterility or the impact of LT at different stages of anther/pollen development and on microsporogenesis, microgametogenesis, and tapetum degeneration remained largely unexplored with the exception of reports on pollen structure deformities and hypertrophy of tapatum under LT (Kumar et al., 2010) It appears that anthers of tolerant chickpea under LT produce viable pollen by upregulating triacylglycerol and carbohydrate metabolism and by regulating specific set of genes that are involved in pollen development (Sharma & Nayyar, 2014). In other crops, LT disrupts carbohydrate pool in anthers of susceptible plants as a result of abscisic acid-induced downregulation of tapetum cell wall bound invertases and monosaccharide transporter genes, thereby resulting in pollen sterility (Oliver et al., 2005; Oliver, Dennis, & Dolferus, 2007; Sharma & Nayyar, 2016). Based on 50% pollen sterility under LT, two most sensitive stages in chickpea, that is, 9 days and 4–6 days before anthesis were reported (Clarke & Siddique, 2004).

Major lacuna that inhibits elucidation of flower and anther/pollen development regulation in chickpea or identification of flower/anther components that are targeted during the abiotic stresses is the paucity of description of flower and anther development landmarks called as stages. Maiden study on flower development in chickpea dates back to 1968 (Eshel, 1968) wherein five flower development stages, (a) closed bud, (b) hooded bud, (c) half open flower, (d) fully opened flower, and (e) faded flower, were described. Similarly, a preliminary framework on pollen development in chickpea was described by Clarke and Siddique (2004). Significant progress has been made in understanding flower and anther development in model plant Arabidopsis (Sanders et al., 1999; Smyth, John, Bowman, & Meyero witz, 1990) as well as in some other crops such as tomato (Brukhin, Hernould, Gonzalez, Chevalier, & Mouras, 2003 for flower) and wheat (Browne, Iacuone, Li, Dolferus, & Parish, 2018, for anther). The development landmarks described in these studies can form the basis to describe flower and anther development stages in chickpea. Advent of tools for sequencing and gene regulation enabled studies on regulation of anther/flower development in plants including chickpea under abiotic stresses (Sharma & Nayyar, 2014; Singh, Garg, & Jain, 2013). The studies in chickpea, however, appear to be of limited utility in identifying genes regulating specific anther development events, for example, meiosis, tetrad formation, or anther dehiscence as tissues were not harvested at specific anther development stages owing to lack of adequate flower development descriptions. It has already been demonstrated in *Arabidopsis* and rice that different sets of genes govern tissue specification in anthers (Wilson & Zhang, 2009), and hence, targeting the exact development stage is vital to draw meaningful conclusions regarding key male organ development players in chickpea.

In the present study, we describe flower as well as anther/pollen development stages in chickpea and report the impact of LT on male organ development, that is, microsporoegenesis, microgametogenesis, anther dehiscence, and pollen viability. In addition, impact of LT on some female reproductive traits such as ovule viability, stigma receptivity, and pollen load on stigma is also reported. Patterns of recovery of vegetative and reproductive tissues in LT-treated plants that were subsequently grown under normal temperatures (NT) are also described.

2 | MATERIALS AND METHODS

2.1 | Plant materials and growth conditions

Plants of a cold-susceptible chickpea cultivar, GPF2, were grown in pots (diameter: 25 cm top, 18 cm bottom, depth: 25 cm, volume: 9 L, two plants per pot) in a growth chamber (Bhanu Biotech. Pvt. Ltd. Delhi, India, 22 ± 1°C/16 ± 1°C day/night temperature, 50–70% relative humidity, 16 hr photoperiod with ~15,000 lux light intensity provided by cool white fluorescent lamps, the conditions henceforth referred to as NT). At reproductive stage, one set of plants was shifted to LT (4°C day/4°C night maintained in the growth chamber) whereas the other set continued to grow at NT. Except for LT, the other growth conditions were same between LT and NT. The plants growing at NT were used to study flower and anther development stages whereas both sets of plants (NT and LT) were used to study impact of LT on anther and pollen development.

In another experiment, a set of the plants at reproductive stage was transferred to LT for 25 days and then transferred back to NT for subsequent growth and possible recovery. An experiment was also conducted under natural conditions (year 2016–2017, date of sowing October 1, 2016). The growth of plants was observed under LT as well as when the temperatures rose back to normal levels.

All the experiments were conducted using chickpea cultivar GPF2 except for flower development stages experiment that was studied using GPF2 (cold-susceptible) as well as ICC16349 (cold-tolerant).

2.2 | Flower development stages

Buds and flowers of different sizes were tagged and observed for subsequent development till flower opening. Sepal and petal whorls of buds/flowers were also removed to study development of stamens and pistil. Flower stages were selected based on development landmarks. The time for transition from one flower stage to another was also recorded. The flowers were photographed using a Sony alpha 68 DSLR camera 2.8/30 macro lens SAM. Anther length was also measured at all flower development stages using dissecting stereo microscope (Nikon SMZ745T). The anthers after harvesting were placed on moist filter paper discs to avoid desiccation.

2.3 | Anther developmental stages

The buds/flowers at different development stages as well as those between two consecutive stages, for example, 1.5 and 2.5 mm, were fixed in fixative solution of formalin-acetic acid alcohol 1:1:18 for 24 hr followed by dehydration in 70% ethyl alcohol and infiltration in tertiary butyl alcohol series. The tissues were embedded in paraffin wax at 60°C. Serial transverse cross sections (5-μm thick) in at least seven replicates for each stage were generated with Microtome (Bio-craft scientific system, India: Modal No. BMT-01). The sections were stained with saffranin (1% in 50% alcohol) and fast green extra

bluish (0.5–1% clove oil; Johansen, 1940) and were observed under a compound microscope (Nikon eclipse E800).

2.4 | Impact of LT on flower development and growth of LT-treated plants under NT

Plants were grown at NT in a growth chamber (Bhanu Biotech. Pvt. Ltd. Delhi, India) till flower and pod development, flowers of different development stages as well as pods were tagged, and the plants were shifted immediately to LT (growth chamber; Bhanu Biotech. Pvt. Ltd. Delhi, India) for 25 days. Data were recorded on new leaf, bud, flower and pod formation; flower and pod abortion; time of transition from one flower stage to another; anther dehiscence; days to anthesis and pod formation. After 25 days under LT, the plants were shifted back to NT. A set of plants growing at NT acted as control. Data as described for LT were also recorded for other treatments. Same experiment was also conducted under natural conditions at Palampur, Himachal Pradesh, India (GPS Latitude: 32° 07′ 12.00" N, Longitude: 76° 31' 48.00" E), a place with stressful LT to chickpea during brief period in winters (see Figure S1 for temperature range during crop growth period). The crop was sown in pots in October 1, 2016. It was at flowering and podding stage when LT due to winters commenced (first week of January). Growth of plants, shoots, flowers, and pods was recorded during the cold stress period and subsequently when temperatures rose back to normal in February end. Thermal time in degree days (°Cd) was calculated as described by McMaster, Wilhelm, Palic, Porter, and Jamieson (2003). The basal temperature (T_{base}) for chickpea growth was 0°C (Soltani, Hammer, Torabi, Robertson, & Zeinali, 2006).

2.5 | Impact of LT on anther development stages

To study impact of LT on anther development, flower buds at six anther stages, that is, 5 (premeiotic mother cells stage), 7 (tetrad stage), 8 (young microspore stage), 11 (mature pollen stage), 12 (bilocular stage), and 13 (dehiscence stage) were exposed to LT. Histological examination of anthers at four of these stages (5, 7, 8, and 11) was carried out. The flowers after exposure to LT were collected and analysed histologically as described above. The buds/flowers of different stages under LT were harvested at different time intervals that corresponded to time taken by anthers to reach next anther development stages till stage 14c. Time of transition from one development stage to another under NT was also studied. The flowers of Stages 12 and 13 were not analysed histologically but were examined for anther dehiscence, time taken for anthesis, and pollen viability. Each treatment in this experiment had seven replications. Plants growing at NT acted as control.

2.6 | Reproductive biology

Pollen viability, ovule viability, pollen load on stigma, and stigma receptivity were studied. Pollen viability was studied in flowers that were exposed to LT at flower Stages 10, 11, 12, and 13 whereas ovule viability, pollen load on stigma, and stigma receptivity were studied in flowers that were exposed to LT at Stages 12 and 13. The reason for choosing Stages 10, 11, 12, and 13 for pollen viability was lack of mature pollen development under LT in earlier stages, and opting for Stages 12 and 13 for other traits was lack of anther dehiscence under LT in earlier stages. Bright field microscopy (Nikon eclipse E800) was used in this experiment. The experiment had two sets of plants, that is, growing under NT (control) or LT with five replications per treatment for pollen viability and stigma receptivity and 10 replications for each of the other two parameters. There were 10 plants per replication, and at least one flower per plant was used in these experiments.

Pollen viability was estimated using 150–200 pollen grains (1 to 5 microscopic fields). Pollens were stained with 0.5% acetocarmine/Alaxander triple stain (Alexander, 1969). For this experiment, the pollens were collected from flowers at Stage 16. Ovule viability was determined as per Kaushal et al. (2013). Ovules were removed from flowers at Stage 15 and placed into a drop of 2,3,5-triphenyl-2H-tetrazolium chloride (Sisco Research Laboratories Pvt. Ltd., India) solution (0.5% in 1% sucrose solution) on a clean glass slide. The slides were incubated in dark for 15 min under room temperature and ovules examined under bright field microscope (Nikon eclipse E800). Ovule viability was calculated using 1–5 scale (1 = *lowest intensity* and 5 = *high intensity*).

Pollen load was estimated immediately after anther dehiscence (Stage 15). Pollen load on stigma was scored on a 1–5 scale (1 = *low* and 5 = *high*; Srinivasan et al., 1999). To detect stigma receptivity, esterase test was conducted using α-nephthyl acetate as a substrate in the Azo-coupling reaction with fast blue B as per Mattson, Knox, Heslop-Harrison, and Heslop-Harrison (1974). For stigma receptivity, stigmas from flowers at Stage 15 were removed, immersed in a solution containing α-nephthyl acetate and Fast blue B in phosphate buffer, at 37°C for 15 min. The reddish brown colour developed on the surface of stigma was scored on a 1–5 scale (1 = *low receptivity* and 5 = *high receptivity*; Kumar et al., 2010).

2.7 | Statistical analysis

All experiments were conducted in randomized complete block design. The data were analysed as one factorial experiment design using AGRISTAT statistical software (ICAR Research Complex Goa, India). For all the parameters, means were plotted, and the standard error values were calculated. The comparisons were made using the Fisher's least significant value at <0.05 level of significance.

3 | RESULTS

3.1 | Flower development in chickpea

Development of chickpea flowers was described from 0.5-mm bud onward till the flowers were fully open. Flower development was divided into 12 stages (7–18; for descriptions of each stage, see

Table 1, Figure 1a,c, Figure S2 for ICC16349). Early flower development (Stages 7–9) in chickpea (present study) and *Arabidopsis thaliana* (Alvarez-Buylla et al., 2010; Smyth et al., 1990) was almost similar, and an assumption was made that flower development prior to this, that is, Stages 1–6 in chickpea, might be similar to *A. thaliana*. Based on this hypothesis, the first stage (sepal enclosed bud containing stamen primordia) in the present study was named as Stage 7.

Comparison of bud/flower length with flower development stages revealed a direct correlation between the two (Figure 1b) up to Stage 17. Thereafter, the flowers did not elongate. The time of transition from one flower development stage to another was also recorded (Table 1, Table S1). It took 12.4 days for the flower of GPF2 to develop from Stage 8 (1-mm long) to Stage 17 (fully functional flower) and 1 day from Stage 14 to Stage 17.

The study also led to the elucidation of sequence of events by which chickpea ensured pollination. The pollination in chickpea was governed by a series of events ensuring synchronous elongation of stamens and pistil, inception of stigma receptivity, and dehiscence

TABLE 1 Flower development stages in *Cicer arietinum* L.

Flower stage	Bud size (mm)	Landmark event at the beginning of stage	Time in decimal days to reach anthesis (Stage 17)
7	0.5	Sepal enclosed bud containing stamen primordia	Not recorded
8	1	Sepal enclosed bud containing green colour anther locules (431.19 μm)	12.40
9	2	Sepal enclosed bud, petal primordia stalked at the base, anther length = 501.76 μm	10.50
10	3	Sepal enclosed bud, petals visible behind the stamens and are shorter than stamen, gynoecium shorter than stamen, stamen length = 2 mm, anther length = 709.39 μm	7.60
11	4	Sepal enclosed bud, filament length increases, petals level with stamen, stamen length = 3 mm, anther color changes, anthers lead towards maturity, anther length = 729.61 μm, gynoecium shorter than long androecium	5.50
12	5	Sepal enclosed bud, sepals start separating, corolla not visible, stigmatic papillae appear, gynoecium levels with long androecium, stamen arrange in two whorls one whorls is shorter than other showing 5 + 5 arrangement, anther length = 739.07 μm, sepals = 5 mm, petals = 4 mm, stamen (long) = gynoecium = 4 mm	3.50
13	6	Corolla visible from inside the calyx and is slightly below the calayx; sepals, petals and gynoecium increase in length. Gynoecium longer than (about 1 mm) stamens, stigma elongation is underway, anther arrangement is 5 + 4 + 1. Sepals = petals = gynoecium = 6 mm, stamen = 5 mm, anther length = 848.40 μm	2.25
14	7	Corolla clearly visible and is slightly above the calayx; sepals, petals, stamens and gynoecium increase in length; gynoecium longer than (about 1 mm) stamens, anther arrangement is 5 + 4 + 1. Stigma becomes receptive, emasculation could be done at this stage. Sepals = petals = gynoecium = 7 mm, stamen = 6 mm, anther length = 884.05 μm	1.00
15	8	Corolla is longer than calyx. Stamens elongate rapidly and are slightly longer than gynoecium, dehiscence occurs, self-pollination occurs. Keel petal remains closed preventing the entry of foreign pollen. For crossing, pollens should be collected at this stage. Sepals = 7 mm, petals = 8 mm, gynoecium = 7 mm, stamen = 7.5 mm	0.50
16	9	Corolla longer than calyx, stigma elongates rapidly and extends above the long stamens, that is, gynoecium protrudes just above the stamens. Sepals = 7 mm, petals = 9 mm, gynoecium = 8.5 to 9 mm, stamen = 8 mm	0.50
17	10	Hooded flower (anthesis), standard petal starts opening. Stamens elongate rapidly and attain the same height as the stigma. Sepals = 7–8 mm, standard petal = 10 mm, stamen = gynoecium (8.5 to 9 mm)	0
18	10	Fully open flower, standard petal fully expanded, in some cases powdery pollen may be visible outside the keel petals that depicts that the pollination event is complete.	

See Figure 1a for Stages 7, 8, and 18 and Figures 1a,c for rest of the stages.

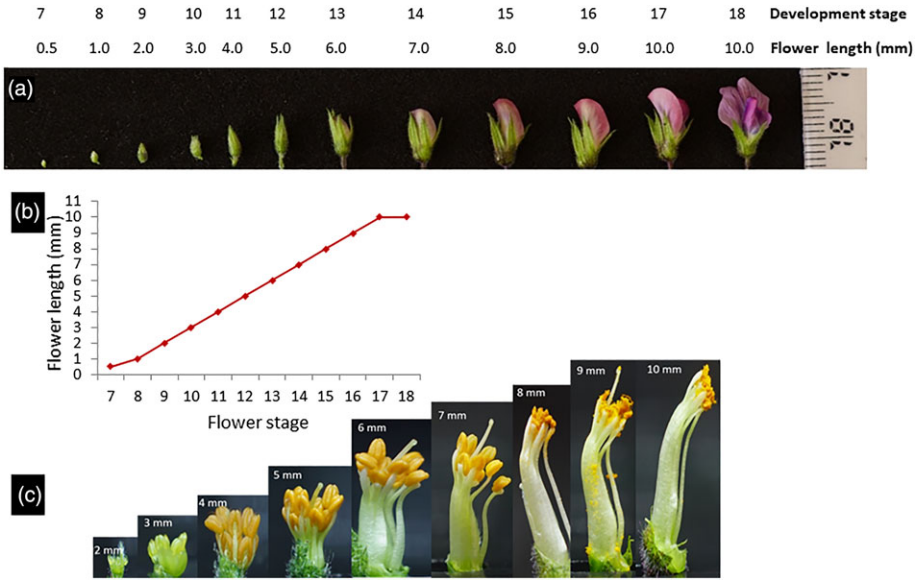

FIGURE 1 Flower development in chickpea (*Cicer areitinum* L.) genotype GPF2 (a and c). The flower development in chickpea was divided into 11 stages (7–18; a). The name of the development stage and bud/flower length in mm are given at the top of (a). There was a direct correlation between flower bud size and flower stages (b) except at Stage 18 as flowers stopped elongating after Stage 17. Arrangement of reproductive parts (stamens and pistil) in chickpea flowers at different stages from Stage 9 to 18 (c) revealed that the elongation and development of stamen and pistil occurred in a synchronous manner so that at dehiscence stage (Stage 15), anthers are slightly above the stigma. Soon after dehiscence, stigma jutted above the dehisced anthers in such a manner that it rubbed through anther whorl ensuring sticking of pollen on its surface (see yellow-coloured pollen grains on stigma in C 9 mm). Soon after pollination stamens grew above the stigma and covered it firmly from all sides [Colour figure can be viewed at wileyonlinelibrary.com]

(see 8–10-mm flowers, Stages 14–17, Figure 1c, and Figure S2). Stigma became receptive at Stage 14 following which the stamens elongated very rapidly to reach above the stigma, anthers formed a tight round whorl above the stigma and released pollen grains (Stage 15). During the process of dehiscence, the keel petal remained closed to prevent the entry of foreign pollen and avoid cross-pollination. Soon after dehiscence, gynoecium elongated rapidly through tight whorl of anthers to about 1 mm above the anthers. During the process of its upward movement, the pollen grains stuck to the surface of the receptive stigma (see pollen load at Stage 16, Figure 1c, Figure S2). The stamens again elongated rapidly and covered the pollinated stigma completely, probably as a protection to stigma till fertilization. Whole of this process (Stages 14–17) took about 24 hr to accomplish, and the flowers opened after 12 hr of pollination (Stages 15–17). The Stage 14 was the appropriate stage for emasculation and Stage 15 for pollen collection during chickpea breeding experiments.

3.2 | Anther development in GPF2 (a cold-sensitive genotype)

3.2.1 | Anther elongation

The anthers did not elongate evenly vis-a-vis flower stages (Figure 2a, b). These elongated rapidly between Stages 9 (anther length 501.76 μm) to 10 (anther length 709.39 μm) and 12 (anther length 739.07 μm) to 13 (anther length 848.4 μm) and slowly between Stages 8 (anther length 431.19 μm) to 9 (anther length 501.76 μm) and Stages 10 (anther length 709.39 μm) to 12 (anther length 739.07 μm; Figure 2a,b). These did not elongate from Stage 14 onward.

3.2.2 | Anther development stages

Chickpea anther development was studied from four lobe anther stage (0.5-mm bud) till anther senescence. The anther development in chickpea was divided in to 11 stages named 4–14 (Table 2, Figure 3). The nomenclature of first stage (Lobe formation stage) as Stage 4 was based on the assumption that earlier developmental stages (Stages 1 to 3) might be similar to those in *A. thaliana*, wheat, and cotton. This assumption originated from the fact that anther development in chickpea (present study) was almost similar to Arabidopsis, cotton, wheat, and rice (Browne et al., 2018; Sanders et al., 1999; Xu, Iacuone, Li, & Parish, 2014; Zhang & Wilson, 2009). The microsporogenesis occurred during Stages 4 to 7, meiosis (I and II) during Stages 5 to 6, microgametogenesis during Stages 8 to 12, anther dehiscence at Stage 13 and senescence at Stage 14. The four locules and sporogenous cells (Sp) within those appeared at Stage 4 (Table 2, Figure 3). The Sp developed subsequently to form microspore mother cells (MMCs; Stage 5), meiotic cells (MCs; Stage 6) and tetrads (Stage 7). The young microspores were released from tetrads at Stage 8 following

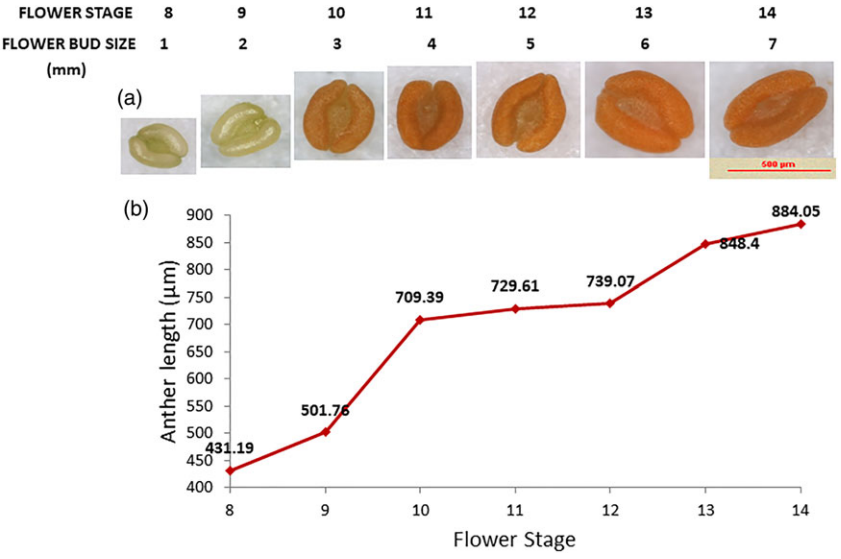

FIGURE 2 Size, morphology, and colour of anthers of chickpea cultivar GPF2 at different flower development stages (a). Correlation of anther length with flower stages or size is presented in (b). Anthers elongated rapidly at flower Stages 9 to 10 (2–3-mm bud size) and at flower Stages 12 to 13 (flower size 5–6 mm) compared with other stages. There was no further increase in anther length beyond Stage 14 (7-mm flower) as anthers dehisced at Stage 15 (8-mm flower). Bar = 500 µm [Colour figure can be viewed at wileyonlinelibrary.com]

degradation of callose layer surrounding tetrads. Middle layer also degraded at this stage. The tapetum first appeared at Stage 5, was at its largest at Stage 8, its degeneration started at Stage 9, and it was degenerated completely by Stage 11. The vacuoles formed in microspores at Stage 9, and pollens started towards maturity during Stage 10. By Stage 11, pollens were fully mature and contained starch grains. The septum degenerated at Stage 12 leading to form bilocular anthers. The dehiscence took place at Stage 13 due to stomium degradation. Anther locules shrank thereafter leading to pollen release (senescence stage: 14 a, b, and c). The anthers eventually fell off from the filaments.

3.2.3 | Flower and anther stage prediction

The anther development was strongly and positively correlated to flower length and anther length in GPF2 as well as in ICC 16349 (Figure 4a–d) implying that these two traits can be used to predict the accurate anther development stages in chickpea. Between these two, flower length measurements are easy, less time consuming, and do not need sophisticated equipment like microscope for micrometry/length measurements as compared with anther length measurements. Flower length also predicts accurately the flower stages. Hence, we recommend the use of flower length to predict flower as well as anther stages for various experiments involving chickpea.

3.3 | Impact of LT on flower development and recovery in LT treated cold-susceptible (GPF 2) plants under NT

LT (4°C) inhibited vegetative as well as reproductive growth in genotype GPF2. Already existing shoots stopped growing. The buds at flower Stages 8 to 11 showed no further signs of development whereas those at Stages 12 onward did grow further albeit at a much slower rate compared with flowers at NT. Flowers of Stages 12, 13, and 14 took 66.50, 42.75, and 19.00 degree days, respectively, under NT for flower opening whereas those under LT took 86.0, 55.20, and 32.80 degree days, respectively (see Table 3). Similar to flower opening, LT also delayed anther dehiscence (57.00, 33.25, and 9.50 degree days, respectively, under NT for Stages 12, 13, and 14; 68.00, 37.20, and 14.80 degree days, respectively, under LT). LT up to flower Stages 11 (anther stages below 10), thus, inhibited dehiscence and flower opening whereas delayed these traits in flowers of Stages 12 (anther Stage 10) onward. The flowers of all development stages aborted under LT (Figure 5a). Although slow flower development was possible from Stage 12 onwards, ultimately these flowers also failed. The pods except those in grain filling stage also turned brown and either aborted or did not grow further or develop seed (Figure 5b). The pods with seeds showed delayed growth but no abortion (Figure 5b).

To study the behaviour of LT-treated plants after recovery (re-exposure to NT), the GPF2 plants, after 25 days of LT treatment, were exposed to NT. Under re-exposure, the plants resumed growth as evidenced by elongation of preexisting shoots that subsequently bore new leaves, viable flowers, and pods (Figure 5c). From the axils of aborted flowers and pods, axillary shoots emerged, obviously from activation of axillary buds, within 10 days of transfer to NT, followed by emergence of buds by 20 days (Figure 5a,b). Some of the axillary branches also bore flowers (see Figure 5c).

Same experiment was also conducted under natural conditions at Palampur, Himachal Pradesh, India (see Figure S1 for temperature profile during crop growth period), and essentially the same results were obtained (Figure 6). The temperature was conducive for chickpea growth except from January to mid-February 2017 when temperature was low. January 7 to 19 was the coldest period (minimum temperature < 4°C; maximum temperature < 15°C except

TABLE 2 Developmental stages of chickpea anther

Anther stage[a]	Bud length (mm)	Flower stage	Stage name[a,b]	Description	Tissues present[c]	Equivalent stages in Arabidopsis (Sanders et al., 1999)	Equivalent stages in rice (Zhang & Wilson, 2009)	Equivalent stages in wheat (Browne et al., 2018)
4	0.5	7	Lobe formation stage	Four lobed anthers arise, each lobe contains all the layers i.e. endothecium, middle layer, sporogenous cell vascular tissue etc., development of stomium region	E, En, T, ML, T, Sp, C, V	4	5	5
5	1	8	MMC stage	Four clearly defined anther locules, MMCs (microspore mother cells) present	E, En, T, ML, MMC, C, V	5	6	6
6	1.5	-	Meiotic cell stage	Anthers increase its size, MMCs undergo meiosis to form meiotic cells, tapetum continue to grow and becomes vacoulated	E, En, T, ML, MC, C, V	6	7	7
7	2	9	Tetrad stage	Meiosis complete and MC develop to form Tds (tetrads). Middle layer degenerates, tapetum becomes more visible	E, En, T, ML, Tds, C, V	7	8a and 8b	8
8	2.5	-	Young microspore stage	Release of young microspores as the callose layer surrounding Tds degrades	E, En, T, YMSp, C, V	8	9	9
9	3	10	Vacuolated microspore stage	Anthers grow longer, microspores become vacuolated, tapetum layer degeneration starts as it moves towards thinness	E, En, T, MSp, C, V, Sm	9	10	10
10	5	12	Vacuolated pollen stage	Tapetum layer becomes thin and uneven, vacuolated pollen leads to maturity	E, En, T, MSp, C, V, Sm	10	11	11
11	6.5	-	Mature pollen stage	Mitotic divisions occurs within the maturing pollen, tapetum degradation, mature pollen becomes round and starch filled, fibrous bands appear in endothecium and connective cells, stomium differentiation begins	E, En, T, PG, C, V, Sm, St	11	12	12
12	7	14	Bilocular stage	Anther becomes bilocular due to degradation and breakage of septum, pollen grains fully mature. Anther is at its maximum length.	E, En, PG, C, V, St	12	13	13
13	8	15	Dehiscence stage	Stomium region degrades, locules open, pollens release	E, En, PG, C, V	13	14	14
14	9–10	16–18	Senescence stage	Stamen senescence takes place, shrinkage of anther, pollen release	E, En, C, V	14 (a, b and c)	Not included in rice description	15

[a]See Figure 3 for photographs of anther stages.

[b]Anther stage names as per Browne et al., 2018 with modifications.

[c]C, connective tissue; E, epidermis; En, endothecium; MC, meiotic cells; ML, middle layer; MMC, microspore mother cell; MSp, microspore; PG, pollen grain; Sm, septum; Sp, sporogenous cells; St, stomium; T, tapetum; Tds, tetrads; YMSp, Young microspores; V, vascular

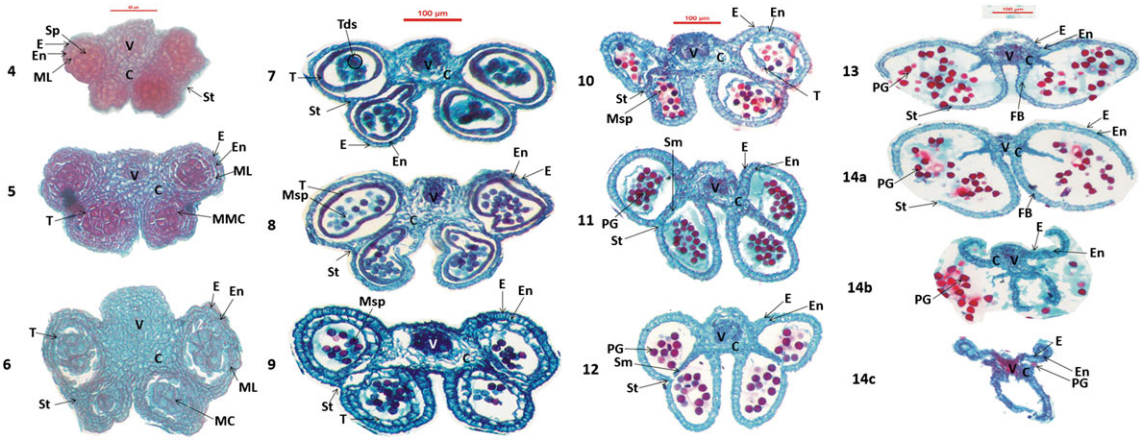

FIGURE 3 Anther development stages in chickpea cultivar GPF2. Flowers were fixed and embedded in paraffin wax and sliced into 5-µm transverse sections as described in Section 2. The flower sections were stained with saffranin, and fast green and anthers were photographed by bright-field microscopy. Stages 4 to 9, 10 to 11, 12 to 13, and 14a to 14c represent anther early development, anther late development, dehiscence, and senescence stages, respectively. Stage names are given on the left of each photograph. C, connective tissue; E, epidermis; En, endothecium; MC, meiotic cell; ML, middle layer; MMC, microspore mother cells; MSp, microspores; Sp, sporogenous cells; T, tapetum; Tds, tetrads; V, vascular region; Fb, fibrous bands; PG, pollen grains; Sm, septum; St, stomium. Stages 4 to 6 bar = 50 µm, stages 7-14c bar = 100 µm [Colour figure can be viewed at wileyonlinelibrary.com]

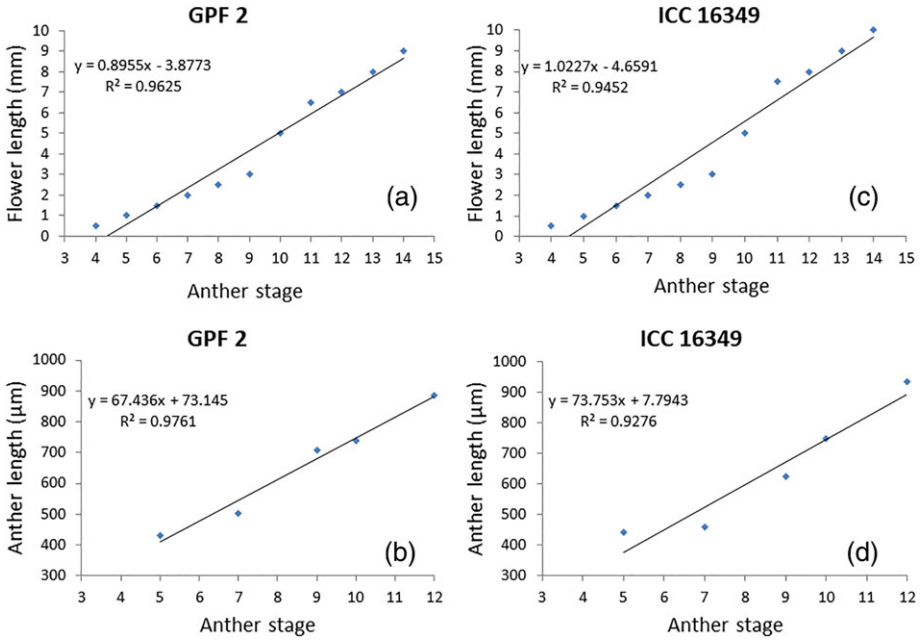

FIGURE 4 Correlation of anther stage with flower length (a, c) and anther length (b, d) in chickpea genotypes GPF2 (a, b) and ICC 16349 (c, d). Anther stages are numbered as per Table 2. Linear regression lines are shown for each graph [Colour figure can be viewed at wileyonlinelibrary.com]

January 16 when temperature was 15.5°C; Figure S1). The plants were already at flowering and podding stages when temperatures dipped to stressful levels in January. Under cold stress, the shoot growth ceased, new buds or flowers did not develop, and those already developed aborted, irrespective of the development stage (Figure 6a). Pods at the beginning of seed filling or younger also aborted (Figure 6a). When temperatures rose back to normal, the plants resumed shoot growth; the shoots started elongating and developed fresh leaves, flowers and pods (see Figure 6b for pod growth under NT). From the axils of leaves bearing aborted flowers or pods, axillary shoots having considerably shorter internodes (3–5 mm) than those in plants at NT (15–17 mm) emerged. These shoots bore flowers and pods primarily from second node onward (Figure 6c,d).

TABLE 3 Thermal time in degree days (°Cd) taken by chickpea flowers (genotype GPF2) to reach anthesis (flower Stage 17) at normal temperature (22 ± 1°C day/16 ± 1°C night temperature) and at stressful low temperature (4°C)

Flower stage	Bud size (mm)	Thermal time (°Cd) to reach anthesis		
		Normal temperature	Low temperature[a]	
		Thermal time[b]	Thermal time	Time in decimal hours[c]
8	1	235.6 (297.60)	No anthesis	No anthesis
9	2	199.5 (252.00)	No anthesis	No anthesis
10	3	144.4 (182.40)	No anthesis	No anthesis
11	4	104.5 (132.00)	No anthesis	No anthesis
12	5	66.5 (84.00)	86.0	516.00 (21.50)
13	6	42.75 (54.00)	55.2	331.20 (13.80)
14	7	19 (24.00)	32.8	196.80 (8.20)
15 or 16	8 or 9	9.5 (12.00; dehiscence)	18.0	108.00 (4.50; dehiscence)
17	10	0 (Anthesis)	0 (Anthesis)	0 (Anthesis)

[a]Flowers of Stages 8–11 stopped growing under low temperature and did not dehisce or underwent anthesis.

[b]Time in decimal hours within parenthesis.

[c]Time in decimal days within parenthesis.

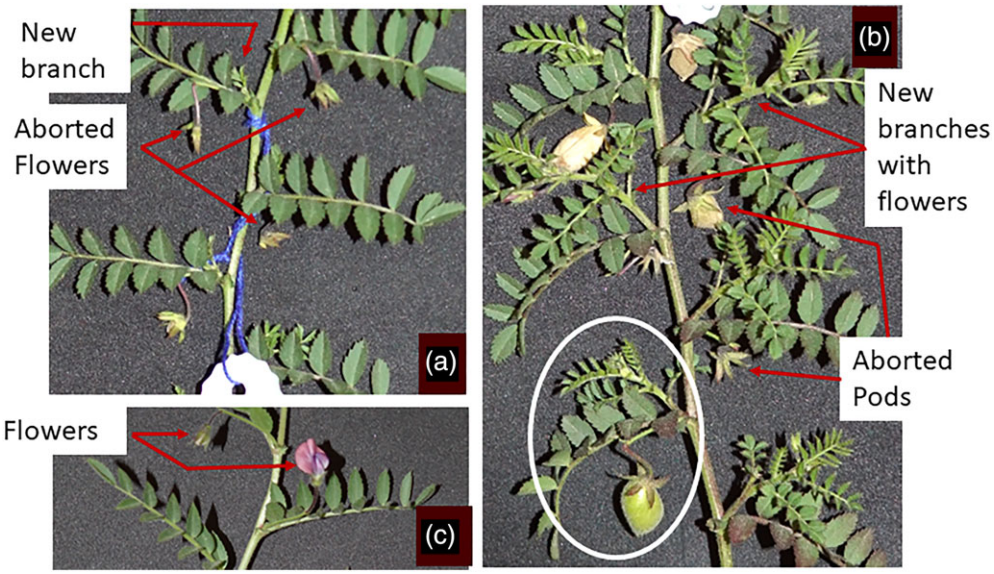

FIGURE 5 Impact of low temperature (LT, 4°C, 25 days) on flower and pod development in cold-sensitive chickpea cultivar GPF2. Under LT, plant growth stopped, all flowers (a) and unfilled pods (b) aborted; however, fully developed pods (see circle in b) did not abort. Re-exposure of LT-treated plants to normal temperature (NT, 22°C day/16°C night) led to the resumption of growth of existing shoots including leaf and flower formation (c). In addition, axillary shoots emerged from axillary buds at the base of aborted flowers (a) and aborted pods (c). These shoots bore flowers after about 20 days of re-exposure under NT (c). Axillary shoots also developed from the axils of fully developed pods that (pods) did not abort (see circle in b) [Colour figure can be viewed at wileyonlinelibrary.com]

3.4 | Impact of LT on anther and pollen development in GPF2

The impact of LT on anther development varied depending upon the anther stage at the time of exposure. LT at premeiotic stage (MMC stage or anther Stage 5) inhibited microsporogenesis as the growth of anthers was arrested at MC stage (anther Stage 6), and tetrads were not formed (Figure 7a). Development of MC from MMC pointed towards initiation of meiosis, and lack of tetrad formation pointed towards incompletion of this process. LT at postmeiotic stage (tetrad stage, anther Stage 7) inhibited microgametogenesis. LT at this stage led to the degradation of anther structures including tapetum within locules of 60% of anthers whereas growth in rest of the anthers was arrested at late tetrad stage (callose layers surrounding tetrads were partially or fully degraded, but microspores were not released; Figure 7b). LT at young microspore (MSp) stage, inhibited tapetum

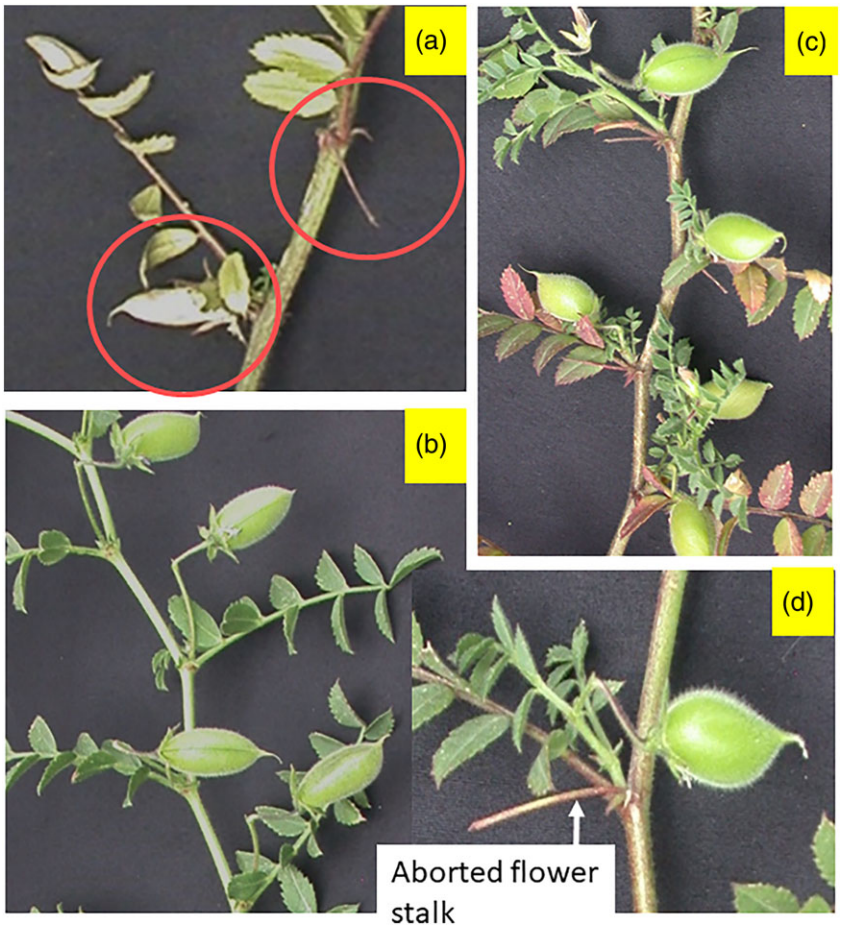

FIGURE 6 Impact of naturally prevailing low temperature (LT) at Palampur, India on flower and pod development in cold-sensitive chickpea cultivar GPF2. Under LT, the flowers and unfilled pods aborted (a, aborted flower stalk and pod shown in circle); however, exposure to normal temperature (NT) after LT-period led to resumption of shoot and flower development (b, c, d). These flowers subsequently grew to form seeded pods (b). At NT after LT, the axillary shoots emerged from axillary buds at the base of aborted flowers and pods (c, d). These shoots bore flowers and pods after about 30 days of growth under NT (c, d). (d) shows the close up of the recovery under NT highlighting an aborted flower stalk, newly emerged shoot, and a pod at the newly formed shoot [Colour figure can be viewed at wileyonlinelibrary.com]

programmed cell death as tapetum did not degenerate, though, the young MSp, grew further to form vacuolated microspores, vacuolated pollen, and mature pollen (Figure 7c). The septum and stomium remained intact with no breakage ultimately resulting in no dehiscence. LT at mature pollen stage (anther Stage 11) delayed degeneration of septum and stomium (Figure 7d), which delayed anther dehiscence. Visual observations on flower development including anther dehiscence under LT (for details, see Table 3 and section "Impact of LT on flower development and recovery in LT treated cold-susceptible (GPF 2) plants under NT") also support these results.

LT also induced pollen sterility (young microspore stage [flower stage between 9 and 10]: no viability, vacuolated microspore stage (flower Stage 11): 23.59% viability, vacuolated pollen stage [flower stage 12]: 52.4% viabity, mature pollen stage [flower stage 13]: 65.5% viability, control: 78.3% viability; Table 4, Figure 8a). Pollen, thus, became increasingly tolerant to LT stress as anther development matures.

3.5 | Effect of LT on female reproductive traits

Ovule viability, stigma receptivity, and pollen load on stigma were studied for flower stages that underwent dehiscence and anthesis (flower Stages 12 and 13). All the parameters were severely affected under LT (Table 4, Figure 8). The impact of LT on ovule viability was more severe than that observed for pollen viability (Table 4, Figure 8 a,b). Ovule viability under LT was reduced to 1.5 and 1.7, respectively at flower Stages 12 and 13 as compared with 4.0 at NT (Table 4, Figure 8b). Unlike pollen viability that was more at Stage 13, the ovule viability was statistically similar at both the stages (Table 4). LT also reduced drastically the stigma receptivity in flowers under LT (Stage 12: 1.3; Stage 13: 1.4) compared with those under NT (3.6; Table 4, Figure 8c). Similarly, pollen load on stigma was also reduced drastically (under NT: 3.7; under LT: Stage 12 = 1.1, Stage 13 = 1.4) with no significant differences between the two stages (Table 4, Figure 8d).

4 | DISCUSSION

The present study describes the major morphological events (landmarks) of flower development in chickpea from stamen primordia stalked at the base to fully opened flower. Early flower development from formation of flower primordium to stamen primordia stalked at the base is yet to be described. The current description, unlike Eshel (1968) who described five flower development stages in chickpea, is based on well-defined morphological landmarks that were described for crops like Arabidopsis (Alvarez-Buylla et al., 2010; Smyth et al., 1990) and tomato (Brukhin et al., 2003). The pattern of flower development in chickpea is similar to that described in Arabidopsis (Smyth et al., 1990), though flower of chickpea is bigger than Arabidopsis. We also defined the sequence of events leading to pollination in

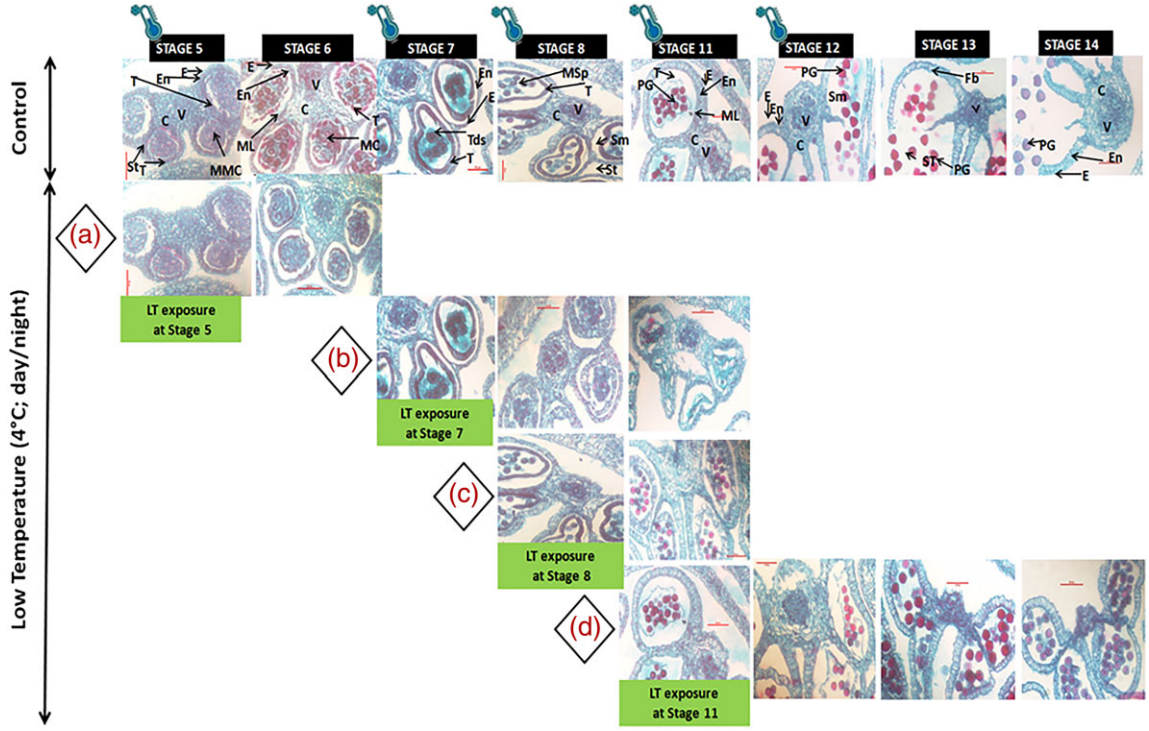

FIGURE 7 Transverse sections of anthers showing impact of low temperature (LT) on anther/pollen development. Plant were exposed to LT at anther Stages 5 (a), 7 (b), 8 (c), and 11 (d). For stage 5, the subsequent development froze at meiotic cell stage (Stage 6; a). For Stage 7, some anthers showed development defects/deformities whereas in others growth was arrested at late tetrad stage (b). Impact of LT at Stage 8 was characterized by ectopic persistence of tapetum layer around pollen grains that developed normally but with increased sterility (c) and no anther dehiscence. LT at Stage 11 interfered with septum and stomium degradation (d), and the anthers did not dehisce. Bar = 50 μm. Stage 5, MMC stage; Stage 6, meiotuc cell stage; Stage 7, tetrad stage; Stage 8, young microspore stage; Stage 11, mature pollen stage; Stage 12, bilocular stage; Stage 13, dehiscence stage; Stage 14, senescence stage [Colour figure can be viewed at wileyonlinelibrary.com]

TABLE 4 Comparison of different reproductive parameters at normal temperature (22 ± 1°C day/16 ± 1°C night) and low temperature (4°C) in cold-sensitive chickpea genotype GPF2

Parameters	Control[a]	Low temperature (4°C)[a]		LSD ($p < 0.05$)
		Flower stage 12[b]	Flower stage 13[b]	
Pollen viability (%)	78.3 ± 1.02 a	52.4 ± 3.5 c	65.5 ± 2.23 b	6.9
Ovule viability (1–5 scale)	4.0 ± 0.13 a	1.5 ± 0.12 b	1.7 ± 0.12 b	0.35
Stigma receptivity (1–5 scale)	3.6 ± 0.22 a	1.3 ± 0.07 b	1.4 ± 0.13 b	0.44
Pollen load on stigma (1–5 scale)	3.7 ± 0.22 a	1.1 ± 0.09 b	1.4 ± 0.14 b	0.45

Note. LSD: least significant value.

[a]Mean ± SE, values with same letter are statistically insignificant ($p \leq 0.05$).

[b]Flower stage at the time of exposure to low temperature.

chickpea. The growth of flower as a whole and stamen and gynoecium in particular was remarkably fast after pollen maturation. The time gap of 12 hr between dehiscence and flower opening was almost similar to that of 15 hr reported by Malti and Shivanna (1983) whereas time gap of 1 day between pollen maturation and flower opening was similar to that reported by Clarke and Siddique (2004). We also demonstrated that chickpea flower development stages have a direct linear correlation with flower length implying that flower elongation is synchronous to changing flower development landmarks and can be used to predict flower development stages. In contrast to this, chickpea anthers elongated irregularly, and anther length was not correlated as linearly as the flower length and, hence, was inferior to flower length for prediction of flower development.

Anther development from "lobe formation stage" onward was described. The present study elaborated the preliminary framework of pollen development described by Clarke and Siddique (2004) to describe well-defined anther development stages in chickpea. Anther development in chickpea was almost similar to that in Arabidopsis, cotton, rice, and wheat (Browne et al., 2018; Sanders et al., 1999; Xu et al., 2014; Zhang & Wilson, 2009). The names of anther

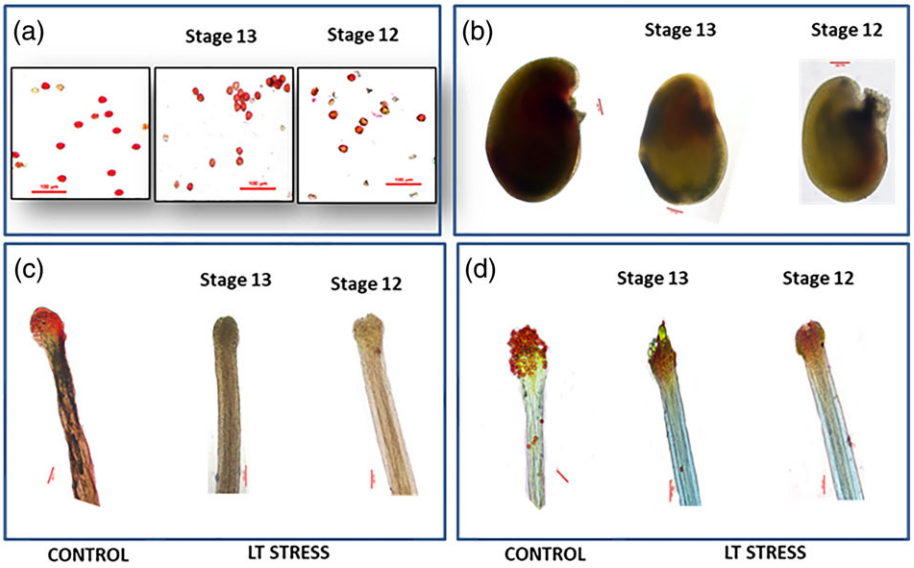

FIGURE 8 Effect of low temperature (LT, 4°C) on pollen viability (a), ovule viability (b), stigma receptivity (c), and pollen load on stigma (d) in chickpea cultivar GPF2. Plants were exposed to cold stress at flower development Stages 12 and 13. Control, that is, normal temperature (22 ± 1°C [day]/16 ± 1°C [night]) treatments are on extreme left in a, b, c, and d. LT reduced pollen viability, ovule viability, stigma receptivity, and pollen load on stigma. Bar = 100 μm [Colour figure can be viewed at wileyonlinelibrary.com]

development stages described in this study were adopted from Browne et al. (2018, wheat) and were modified to describe chickpea anther development. We also described a non-destructive and cost-effective method based on flower length to predict anther development stages in chickpea. This method will provide a convenient tool for assessing anther development stages in chickpea without anther sectioning. Similar to present study, spikelet length as well as anther length in wheat correlated with anther development stages (Browne et al., 2018). The flower and anther development stages will form the basis to identify gene regulatory networks in chickpea floral organ differentiation and development including anther and pollen development. In addition, precise description of anther development can also be used as a guide for selecting correct anther age for cross-pollination or further research on the effects of abiotic stresses on pollen development and anther dehiscence (as done in the present study). Since, male organ development is a complex phenomenon involving premeiotic and postmeiotic events and anther dehiscence (Zhang & Wilson, 2009; Wilson & Zhang, 2009; present study), and cold affects chickpea anther growth in an age dependent manner (present study); precise descriptions of floral/anther development will allow exactitude in understanding the effects of LT in chickpea. Such descriptions, if available earlier, Singh et al. (2013) and Sharma and Nayyar (2014) might have focussed on specific stage(s) and reported genes involved in floral development or LT-tolerance at specific anther development events such as meiosis, tetrad formation, etc. In addition to it, the study also has implications in identifying key players involved in abiotic stress tolerance. This information can subsequently be used in abiotic stress tolerance breeding in chickpea.

LT stress induced flower abortion in chickpea was earlier attributed primarily to reduction in pollen viability (Clarke & Siddique, 2004; Kaur et al., 2011; Kumar et al., 2010). The present study, however, showed that pollen sterility was a major but not the sole male factor responsible for flower abortion under LT. LT affected male gamete development in a flower/anther age dependent manner where outcome ranged from no pollen formation to pollen sterility or no dehiscence/delayed dehiscence, and the causes of flower abortion varied with the flower stage (see Figure 9 that summarises data on anther/pollen development from several experiments in the present study). Until anther Stage 10, flower abortion can be attributed to male gamete factors as no pollen was available for fertilization (up to anther Stage 7: no pollen formation, between anther Stage 8–10: no dehiscence). As the flower moved towards pollen maturity (anther Stage 11), LT did not inhibit pollen formation but caused pollen sterility and delayed anther dehiscence (Figure 9) resulting probably the nonsynchronization of stigma receptivity and pollination. Reduction in pollen load on stigma under LT may either be due to nonsynchronization of stigma receptivity and pollination or reduced stigma receptivity or both. Reduced ovule viability further indicated that chances of fertilization might be poor even if pollen tubes reach the ovules, a fact vindicated by abortion of flowers at anther Stages 12, 13, and 14 that bore sufficient viable pollen. In addition to factors reported here, defects in pollen germination, pollen tube growth, and changes in ovule size and ovule fertilization (Clarke & Siddique, 2004; Srinivasan et al., 1999) might be the other contributors of floral abortion under LT. Reduction in ovule viability, stigma receptivity, and pollen load on stigma under LT as observed in the present study has been reported widely in chickpea (Kaur, Kumar, Nayyar, & Upadhyaya, 2008; Kumar et al., 2010; Nayyar, Bains, et al., 2005; Srinivasan et al., 1999). In contrast to these studies, Clarke and Siddique (2004) found no differences for exudate formation on stigma and pollen load at 12°C/7°C (day/might) and at NT except for delayed development. LT also causes changes in ovule structure and ovule viability in other

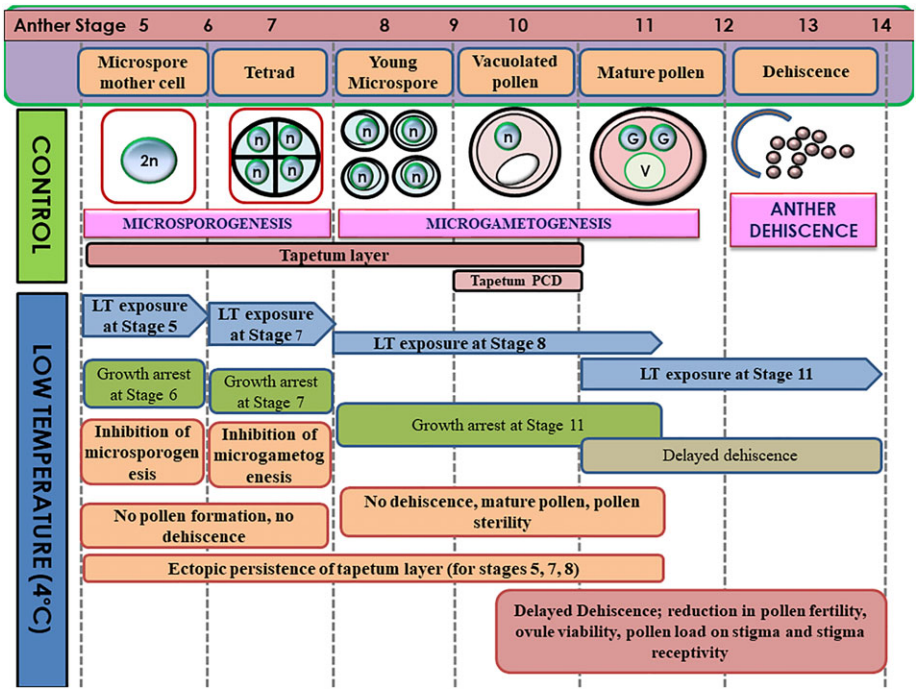

FIGURE 9 Overview of the effect of low temperature (LT) on anther/pollen development and female gamete traits in chickpea. LT prior to meiosis (anther Stage 5, meiotic mother cell stage) inhibited anther growth at meiotic cell stage (anther Stage 6) whereas LT at tetrad stage (anther Stage 7, immediately after meiosis) led to degradation of locule content in 60% of the anthers and growth arrest in rest of the anthers as tetrads did not separate to release young microspores. LT exposure at young microspores stage (anther Stage 8) did not inhibit pollen development as young microspores grew to form mature pollen grains (anther Stage 11) but inhibited anther dehiscence, and pollen was sterile. LT at these three stages interfered with tapetum programmed cell death as tapetum persisted and did not degenerate even when pollen were mature and at Stage 11. LT at vacuolated pollen stage and onward (anther Stage 10 onward) delayed anthers dehiscance (due to delayed breakage of septum and stomium), induced pollen sterility and reduced ovule viability, stigma receptivity, and pollen load on stigma. The pollen sterility decreased with pollen maturity as it was higher when young microspore were exposed to LT and reduced gradually till Stage 13. The conclusions presented in the figure were drawn from Figures 3, 7, and 8 and Tables 3 and 4 [Colour figure can be viewed at wileyonlinelibrary.com]

crops (Ebadi, May, Sedgley, & Coombe, 1995; Lardon & Triboi-Blondel, 1994). Lack of pollen formation under LT was due to failure of microsporogenesis or microgametogenesis in LT-treated anthers (Figure 9). Hypertrophy of tapetum under LT as observed by us was also reported by Kumar et al. (2010) in buds of <1 to 3-mm size. Inhibition of tapetum degeneration under LT might be the cause of inhibition of microsporogenesis, microgametogenesis, and pollen sterility in chickpea (present study) as tapetum programmed cell death had already been demonstrated to be essential for microsporogenesis, male gametogenesis (Li et al., 2011; Vizcay-Barrena & Wilson, 2006), and any deviation in tapetum programmed cell death induces male sterility (De Storme & Geelen, 2014; Jung et al., 2005; Oliver et al., 2005; Sorensen, Guerineau, Canales-Holzeis, Dickinson, & Scott, 2002). Inhibition of anther dehiscence under LT is also reported earlier in chickpea (Srinivasan et al., 1999), and the present study demonstrated that this inhibition was due to LT induced nonbreakage of septum and stomium. Using pollen viability as a criterion in chickpea plants exposed to 3°C, two most sensitive periods of cold stress for pollen development in chickpea were reported to be 9 days before anthesis and 4–6 days before anthesis (Clarke & Siddique, 2004). The most sensitive stages to LT are also described in other crops (Sharma & Nayyar, 2016), for example, tomato (11.2 and 5.6 days before anthesis; Patterson, Mutton, Paull, & Nguyen, 1987), soybean (3–4 and 12.5 days before anthesis; Ohnishi, Miyoshi, & Shirai, 2010), rice (tetrad to early uninucleate stage; Oliver et al., 2005), and brassica (tetrad to early uninucleate stage; Yu et al., 2016). In chickpea, it is difficult to assign a particular time of flower/anther development as more sensitive to LT than others primarily because flowers remain sensitive to LT throughout their development, though, mechanisms of flower abortion vary with flower stage. If we confine our focus only to male gamete, then the most sensitive stages to LT are below anther Stage 10 (flower Stage 12) because at these stages, either pollen do not develop or anthers do not dehisce. Consequently, there is no pollen for fertilization.

A unique property of growth resumption after LT treatment was also observed (present study). Shoot induction from axillary buds that bear flowers and pods (with seeds) at shorter internodes after the cold stress is over indicated that chickpea can probably compensate for cold-induced yield losses to some extent if cold period is short and is followed by temperatures suitable for chickpea growth and development. Recovery from cold has also been reported for pepper where flaccid shoots formed as a result of cold became normal once the plants were shifted to NT (Airaki et al., 2012).

It can be concluded that the cold-induced flower abortion in chickpea is the outcome of disruption of microsporogenesis, microgametogenesis, tapetum degeneration, anther dehiscence, and

reduction in pollen viability, ovule viability, stigma receptivity, and pollen load on stigma. If temperature remains low for prolonged period (as is the case in our study), plant growth will stop, flowers will drop, and no new flowers will form. This phenomenon can, however, be reversed if plants are re-exposed to NT.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

FIGURE S1 Temperature profile (daily temperature) during chickpea growth period (session 2016–2017) at Palampur, Himachal Pradesh, India

FIGURE S2 Flower development in chickpea (*Cicer areitinum* L.) genotype ICC16349 (A and C). The flower development stages in ICC16349 (7–18) are similar to those in GPF2 except for flower length (A). The name of development stage and bud/flower length in mm are given at the top of A. There was a direct correlation between flower bud size and flower stages (B) except at stage 18 as flower stopped elongating after stage 17. Arrangement of reproductive parts (stamens and pistil) in chickpea flower at different stages from stage 9 to 18 (C)

TABLE S1 Time of transition (in days) from one anther development stage to another in chickpea genotype GPF2 grown at normal temperature (22 ± 1°C day/16 ± 1°C night temperature)

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