Chilling stress effects on reproductive biology of chickpea

Sanjeev Kumar[1], Harsh Nayyar[1]*, RK Bhanwara[1] and **HD Upadhyaya[2]**

1. Department of Botany, Panjab University, Chandigarh 160 014, India

2. International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru 502 324, Andhra Pradesh, India

*Corresponding author: harshnayyar@hotmail.com

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Abstract

Chickpea is sensitive to chilling temperatures (<10°C), especially at its reproductive phase leading to floral abortion. The exact causes of reproductive failures are not fully understood. In the present study, we assessed the cold-induced damage to development and functioning of male and female components by growing an early flowering chickpea genotype ICCV 96029 under warm conditions of the glasshouse (control; average maximum and minimum temperature ≥28/15°C) as well as under cold conditions of the field (average maximum and minimum temperature ≤20/10°C during reproductive phase). Low temperature of the field environment restricted the vegetative growth and delayed all the phenological stages in comparison to control plants. Apart from this, it led to some vegetative aberrations like chlorosis, necrosis of leaf tips and curling of whole leaf. The damage to reproductive stage involved abscission of juvenile buds and flowers and abortion of pods. On the whole, pollen development at young microspore stage appeared to be severely affected in stressed conditions compared to the control conditions. Pollen viability was suppressed during stressed conditions (60%) compared to normal plants (95%). Stigma receptivity, in vivo pollen germination and pollen tube growth were inhibited in the stressed plants. Fluorescent studies showed that the stigma either did not show any pollen load or pollen grains did not germinate on its surface in stressed plants. Even when the pollen grains germinated, the pollen tubes rarely grew beyond the proximal region of the style; mostly the pollen tubes were impaired in their growth and did not reach the ovules leading to failure in fertilization. The egg and secondary nucleus in such ovules ultimately disintegrated without fertilizing and hence no seed formation occurred.

Introduction

Chickpea (*Cicer arietinum*) is one of the leading pulses grown in several parts of the world, especially in India accounting for about 65% of the world's production (FAO 2007). Chickpea lacks cold tolerance and is sensitive to chilling temperatures (>8°C), especially at its reproductive phase (Srinivasan et al. 1998, Bakht et al. 2006). The reproductive structures can withstand temperature of 8°C minimum to 22°C maximum during the coldest period. The most advantageous temperature range for normal flowering, fertilization and seed set is 10 to 14°C average minimum temperature and 25 to 31°C average maximum temperature. Temperature within the chilling range can limit the growth and vigor of chickpea at all phenological stages but is considered most damaging to yield at reproductive stage. In India, chickpea is grown as a winter-season crop. The northern parts of the Indian subcontinent and southern Australia are the most affected regions due to chilling as the temperature is below 15°C at flowering (Srinivasan et al. 1998, Clark 2001, Bakht et al. 2006, Berger 2007). During the reproductive phase, low temperature is detrimental to normal flowering and pod development, which causes prolonged reproductive phase, floral abortion, poor pollen germination, impaired ovule development, failure in pod set and reduction in seed filling that drastically affects the crop productivity (Singh et al. 1993, 1997, Srinivasan et al. 1998, Nayyar et al. 2005). The reproductive phase, especially in early-maturing chickpea genotypes, coincides with low temperature of the surroundings causing abortion of flowers and impairment in pod set (Singh et al. 1993, Kumar et al. 2005). A deep investigation of development of male and female components is required to prove the sensitivity of the developmental stage and actual cause of reproductive failure.

The effects of various abiotic stresses on structural and functional aspects of reproductive phase have been reported in soybean (*Glycine max*) (Gass et al. 1996, Kokubun et al. 2001), rice (*Oryza sativa*) (Imin et al. 2004), pea (*Pisum sativum*) (Guilioni et al. 1997) and chickpea (Kaur et al. 2008). In rice, low temperature during the reproductive stage has been found to cause degeneration of spikelets, incomplete panicle exsertion

and increase in spikelet sterility (Terres 1991). Subsequently, Takeoka et al. (1992) has identified that booting stage in rice is highly susceptible to chilling and the damage at this particular stage of reproductive development causes ~30–40% yield reduction in the temperate grown rice worldwide (Nishiyama 1995, Andaya and Mackill 2003). Cold stress induces flower abscission in soybean (Gass et al. 1996), pigeonpea (*Cajanus cajan*) (Sandhu et al. 2007) and chickpea (Nayyar et al. 2005), but the actual cause of reproductive failure is unclear.

Pollen sterility is a common cause of low fruit set under cold temperatures at flowering in rice, sorghum (*Sorghum bicolor*), strawberry (*Fragaria* sp) and tomato (*Lycopersicon lycopersicum*) (Lin and Peterson 1975, Toriyama and Hinata 1984). Low temperatures may cause deformation of flowers or floral parts (Rylski 1979) leading to functional sterility or the formation of deformed fruit. It was observed that pod set failure and deformation in fruit could be associated with abnormal ovary development (Rylski 1979). Fertilization process, subsequent embryo development and seed filling are potential targets of cold stress. It was observed that under cold conditions, the development of male gametophyte is impaired which leads to smaller pollen grains and an increase in non-viable pollen (Gudkova 1980). Imin et al. (2004) while studying the effects of early cold stress on the maturation of rice anthers demonstrated for the first time that cold temperature stress at the young microspore stage enhances and induces partial degradation of proteins in the rice anthers at the trinucleate stage.

Chickpea can experience yield losses up to 50% due to cold stress (Saxena and Johansen 1990) depending upon the severity of the stress and sensitivity of the genotype. Hence, incorporation of cold tolerance in chickpea cultivars is an important prerequisite for winter sowing of the crop (Singh et al. 1990, Bakht et al. 2006). Generally, some chickpea genotypes escape flowering at low temperature but ICCV 96029, an early-maturing genotype, is able to produce flowers under these conditions but most of them abort due to stress thereby affecting its yield. Therefore, we considered it an ideal chickpea genotype for detailed investigations on reproductive biology. Information on the effects of cold stress on reproductive biology of chickpea is rare and the reasons associated with reproductive failures due to low temperature stress are least understood. Therefore, the present study was focused on: (a) probing the most sensitive stage of the pollen development as affected by cold stress; (b) evaluating the effect of cold stress on pollen-pistil interaction; and (c) investigating whether the male/female part is responsible for reproductive failure.

Material and methods

An early-maturing chickpea genotype ICCV 96029 was employed for the present study. Chickpea seeds were sown on 1 Nov 2003 in earthen pots (12 inches diameter) under glasshouse conditions (warm climate; average temperature ~25/15°C day/night) as well as under field conditions (average temperature ≤20/10°C day/night during reproductive stage) after inoculation with *Rhizobium* culture. The response of plants to the lowest temperature (average temperature ≤10°C) especially during the reproductive phase was observed and photographed. Young buds (<1–3 mm) and flowers (from 1–3 days after anthesis) were collected and fixed from both the temperature regimes and further evaluated for various reproductive development stages of male reproductive part, post-pollination and post-fertilization events. Daily temperature (air) of the experiment fields was recorded with temperature sensors and presented as daily maximum and minimum and average temperature. The temperature profile of the field is presented in Figure 1. Comparative observations were made on the basis of the following parameters.

Visual observations. The plants growing under cold conditions were observed for various morphological changes such as slow vegetative growth, chlorosis, leaf tip burning and appearance of anthocyanins during the coldest period (≤10°C).

Reproductive biology. For investigations on reproductive components, at reproductive stage, the microscopic buds (<1–3 mm) and open flowers were collected 1–3 days after anthesis in the morning from plants growing under both the temperature regimes. The flowers from the field-grown plants were collected when the temperatures during the preceding 3 days remained below 20°C (day)/ 10°C (night) (average ≤10°C) in order to enable the plants to experience chilling injury. The microscopic buds and open flowers were fixed in formalin-acetic acid-alcohol, dehydrated in an ethyl alcohol-tertiary butyl alcohol series and embedded in 60°C melting point paraffin. Serial longitudinal and cross sections of buds and flowers at different stages of development were cut, most of the younger ones at 5 µm but some older ones at 7, 8 or 10 µm with Spencer Rotary Microtome (American Optical Company). The sections were stained with saffranin fast green (extra bluish) (Johansen 1940) and observed under a compound microscope. Micrographs obtained with light microscope (Olympus) were used to record observations.

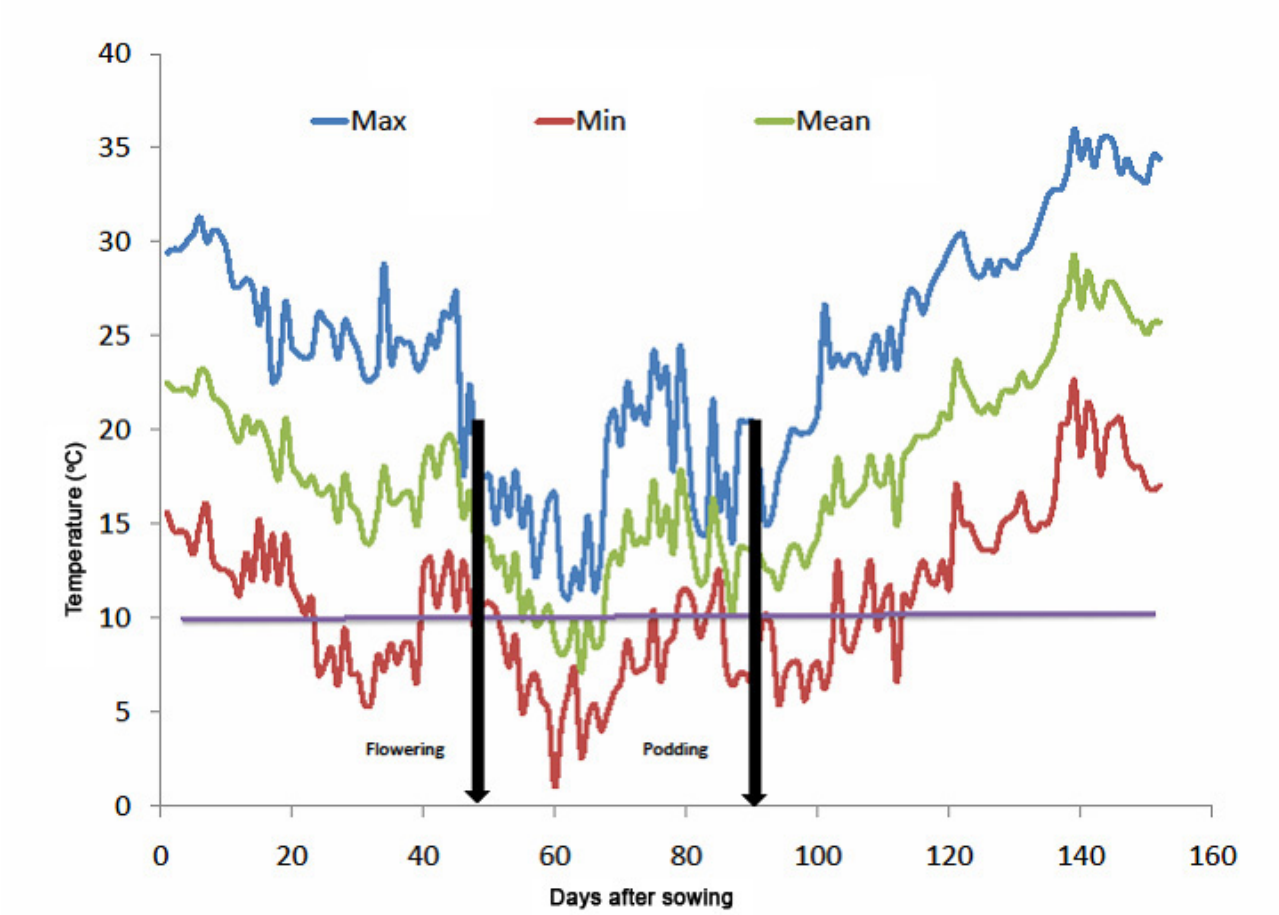

Figure 1. Temperature profile (daily temperature) of the field during reproductive phase of chickpea.

Pollen viability. Pollen viability was tested on 200 pollen grains (5–10 microscopic fields) with 0.5% acetocarmine/Alexander stain. The pollen was collected from the flowers open on the same day. About 3–5 flowers were collected and pollen grains were bulked and thereafter examined for their viability (Alexander 1969). The criteria for selecting viable pollen was size of pollen, shape of pollen (triangular or spherical) and intensity of stain taken up by the pollen.

Pollen morphology using scanning electron microscopy. The pollen grains were observed under scanning electron microscope (SEM) to examine any structural changes. On the day of anthesis, fresh flowers were collected early in the morning from control and stressed plants. Anthers from 10 flowers were collected and teased on a metallic stub. Samples were mounted fresh with double-stick tape, without dehydration, and critical point drying, sputter coated with gold paladium and scanned under SEM (Postek et al. 1980).

Stigma receptivity. Stigma receptivity describes the competence of the stigma to hold pollen grains and allow them to germinate by providing them necessary germination media. To detect stigma receptivity, esterase test was carried out using α-napthyl acetate as substrate in the Azo-coupling reaction with fast blue B as modified by Mattson et al. (1974). Stigmas were removed one day before flower opening, immersed in the working solution containing α-napthyl acetate and fast blue B in phosphate buffer, at 37°C for 15 min. Reddish brown color developed on the surface of the stigma was scored on a 1–5 scale (1 = Low receptivity and 5 = High receptivity).

Pollen germination and pollen tube growth (in vivo). Aniline blue staining was done to assess the pollen germination on stigma and to trace pollen tube in the style and ovary. Flowers were collected on the day of opening or anthesis to consecutive three days after anthesis from field and glasshouse plants and fixed in acetic alcohol (1:3) for 24 h and cleared in 8N NaOH for 6 h at 60°C. The gynoecium was stained overnight with aniline blue solution (0.1% in 0.1 mM Na_3PO_4). The stained gynoecia were mounted on aniline blue : 10% glycerine (1:1) and observed by Epi-Fluorescent method in FLUPHOT microscope (Nikon, Japan) with excitation filter

UV 330-380, dichloric mirror DM-400 and absorption filter 420K (Dumas and Knox 1983).

Phenology and yield. Data pertaining to phenological stages (ie, days to flowering, flowering duration, podding time, podding duration, pod maturity and crop maturity) and to reproductive structures (ie, total flowers produced in a season, flowers abscised, floral retention, pod set percentage and pod retention) were recorded from time to time during the season. Plants were harvested individually at maturity and yield related traits such as total pods, number of seeds, average seed weight, number of one- or two-seeded pods, infertile pods and seed yield per plant were assessed separately (Table 1). The experiment was conducted in randomized complete block design (RCBD) having three replications (15 plants). Data was subjected to statistical analysis and level of significance measured at $P < 0.05$.

Results

Morphological alterations. Plant growth varied under warm and cold conditions. A difference in branching pattern was noticeable; the basal primary branches were significantly more in field-grown (cold) plants as compared to plants grown in the glasshouse (warm) where the number was significantly less. The vegetative growth (ie, plant height) was higher (51.9 cm) under warm conditions compared to the cold conditions (33.4 cm) (Fig. 2A). Similarly, the onset of the reproductive phase was 20 days early under warm conditions as compared to plants grown in the field. In field-grown plants 100% flowering occurred in 1021 degree days whereas glasshouse plants showed 100% flowering in 740 degree days. Flowering duration did not differ significantly between the two conditions whereas total duration of podding reduced significantly (49.2 days) in field-grown plants. The total number of flowers produced per plant (69.2) in field conditions during the season was threefold of that in the glasshouse (23.8), but floral retention was significantly more in the latter (60.5%) compared to the former (33.6%). Though the pod set was significantly lower in the field plants (35.5%) than in the glasshouse plants (44.4%), the former showed appreciably more pod retention (58.9%) than the latter (44%). All the traits contributing to the yield were markedly higher in the field plants than the glasshouse plants. There was no difference between the numbers of infertile pods per plant in the two environments (Table 1).

Table 1. Comparison of growth and yield traits in chickpea genotype ICCV 96029 under warm (glasshouse; control) and cold (field) conditions (Mean ± SEM)[1].

Parameters	Control	Cold stressed
Plant height at 60 DAS (cm)	51.9 ± 1.8	33.4 ± 1.5
No. of basal primary branches at 100 DAS	1.4 ± 0.17	6.0 ± 0.69
Flowering time (days)	37.8 ± 1.5	59.7 ±1.6
Flowering duration (days)	63.6 ± 1.5	65.3 ± 1.8 NS
Podding time (days)	48.2 ± 1.8	93.6 ± 3.2
Podding duration (days)	62.4 ± 1.9	49.2 ± 2.3
Time to pod maturity (days)	92 ± 3.2	120 ± 2.7
Time to crop maturity (days)	113 ± 2.6	138 ± 2.8
Total flowers produced per plant during the season	23.8 ± 4.9	69.2 ± 4.4
Total flowers abscised during the season	9.4 ± 1.3	46 ± 2.0
Floral retention (%)	60.5 ± 2.5	33.6 ± 2.2
Pod set (%)	44.4 ± 0.8	35.5 ± 1.3
Pod retention (%)	44 ± 2.4	58.9 ± 2.2
No. of pods per plant	4.4 ± 0.7	14.4 ± 1.3
Average pod weight (g)	1.0 ± 0.1	3.2 ± 0.3
No. of seeds per plant	4.0 ± 0.7	15.6 ± 1.2
One-seeded pods per plant	2.8 ± 0.6	10.0 ± 1.4
Two-seeded pods per plant	1.5 ± 0.17	2.8 ± 0.34
Infertile pods per plant	2.0 ± 0.3	1.4 ± 0.3 NS
Seed yield per plant (g)	0.6 ± 0.11	2.7 ± 0.4
Average seed weight (g)	0.18 ± 0.02	0.18 ± 0.03 NS

1. DAS = Days after sowing; NS = Not significant.
Source: Kumar et al. (2005)

Anthocyanin accumulation. The stressed plants accumulated anthocyanins in the basal part of the stem, branches and leaves (Fig. 2B, C; arrows) while no such accumulation occurred in control plants. The pigmentation was sustained till the low temperature prevailed and disappeared slowly when the plants experienced subsequent higher temperature (average >15°C) in the field.

Effect on vegetative structures. Generally, field-grown plants did not show any change in vegetative structures. However, some aberrations like chlorosis, necrosis of leaf tips and curling of whole leaf was observed (Fig. 2D; arrow).

Floral abnormalities. Conventionally, solitary flowers exist in majority of cultivars but in ICCV 96029, the phenomenon of floral dimorphism was observed in the plants growing only under cold conditions, ie, a peduncle bearing two pedicellate flowers, one having purple pedicel and the other with green pedicel, unlike in plants grown under warm conditions (Fig. 2E). The purple-pedicellate flower had normal vexillum, keel, stamens and gynoecium, but in contrast, an abnormal floral structure was noticed in green-pedicellate flower with deformed shape, twisted stamens and sometimes these flowers exhibited two sets of stamens and gynoecia suggesting alteration in reproductive structure by cold stress. The flowers with green pedicel finally abort

Figure 2. Morphological and reproductive damage due to cold – A: Growth pattern under warm and cold conditions; B: Anthocyanin accumulation in stressed plants (arrow); C: Anthocyanin accumulation in leaves (arrow); D: Burning of leaf tips (arrow); E: Floral dimorphism; F: Normal flower setting pod (arrow); G and H: Floral abortion (arrows); I: Pod abortion (arrow); J: Normal pod set.

without forming pods (Fig. 2F, G, H; arrows). This situation prevailed until the day (>20°C) and night (>10°C) temperature became normal. In late winters, when the conditions become warm, with the rise in day/night temperature the flowering becomes profuse, solitary with early pod set and early maturity of pods/seeds. The reasons for this abnormality are not known but need to be examined since substantial numbers of the potential pods are aborted under cold stress.

Additionally, we also observed that under cold stress flower development does not start in a normal manner. After anthesis flowers either abscise without forming pods or the pods often abort at different stages of pod/seed development. In rare cases, if the pod develops in a normal manner, it ultimately aborts later without seed filling (Fig. 2I; arrow). In mid winters (mid January to mid February), when day temperature rises (>20°C) but night temperature remains low (<10°C), among two types of flowers, the normal flower having purple pedicel starts setting fruit/pod and seed while the abnormal flower aborts without forming pod (Fig. 2H; arrow). Occasionally, if the flower with green pedicel sets pod, it leads to undeveloped pod and seeds, hence a condition of pod abortion arises (Fig. 2I; arrow). Floral dimorphism observed here was a unique observation in ICCV 96029. The survival of purple pedicellate flowers might be related to the anthocyanin accumulation ability. The plants growing under field conditions showed double healthy pods later in warm climate (Fig. 2J).

Pollen development as affected by cold. In both cold-stressed and control (warm condition) plants, a transverse section of anther revealed four microsporangia with connective tissue in the center (Figs. 3A, B and 4A, B). In control plants, however, the parenchymatous cells of the connective region showed starch granules (SG; Fig. 3A, B) while these were found to be absent in plants under stress conditions (Fig. 4A, B). Each microsporangium in both the temperature regimes comprised an epidermis, endothecial layer, a middle layer and tapetum enclosing microspore mother cells (MMCs) as seen in the transverse section. The tapetal cells were observed to be uni-nucleate with dense cytoplasmic contents (Figs. 3B and 4B). The MMCs were large, polygonal/hexagonal, each with a conspicuous nucleus and a nucleolus (Figs. 3B and 4B). After completion of meiotic divisions, microspores get separated from the tetrad arrangement (stage presented in whole mounts, Fig. 5A, B). The anther wall layers showed some difference in plants under control and stressed (cold) conditions. In control plants, the epidermal cells became stretched and compressed, the endothecial cells showed radial elongation and development of secondary wall thickenings (ie, endothecial thickenings – ET), the middle layers disintegrated and tapetal cells showed sign of disorganization (Fig. 3C, D). On the other hand in the stressed plants, disintegration of wall layers was seen. There was an indication of the development of secondary wall thickenings (ET) but in some cases it was not observed (Fig. 4D, E). Traces of tapetum were still present in older buds showing no sign of disintegration (Fig. 4C, D, E). The young microspores in unstressed plants were normal, spherical cells with dense cytoplasmic content and a prominent nucleus in the center (Fig. 3C, D). On the contrary in stressed plants the young microspores were less in number, distorted in shape and transparent, indicating less cytoplasmic content as a result of abnormal development (Fig. 4D, E).

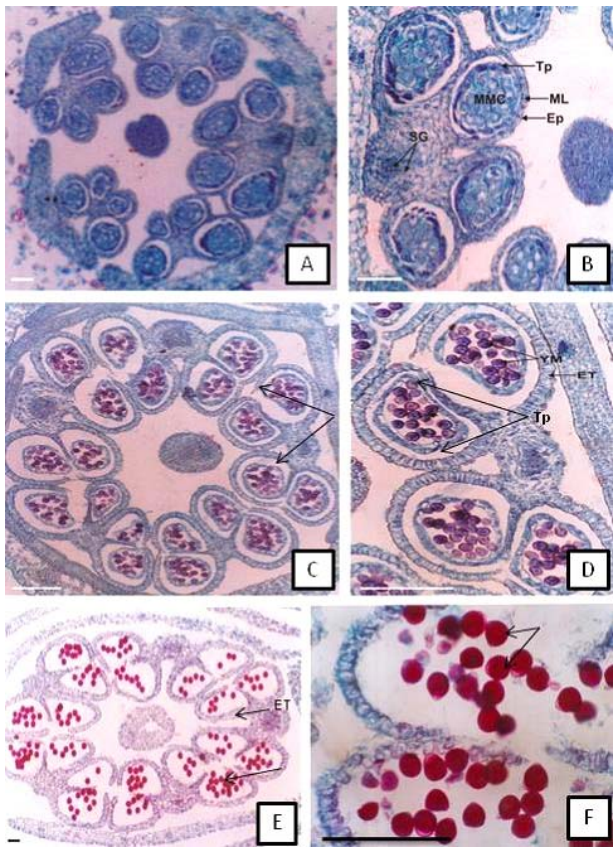

Figure 3. Transverse section (TS) of young microscopic bud of control (warm condition) plants showing different stages of microsporogenesis – A and B: TS of young bud showing tetrasporangiate anther at microspore mother cell stage, with different wall layers (EP = epidermis, ML = middle layer, Tp = tapetum) enclosing microspore mother cell (MMC) and starch granule (SG) at the connective region; C and D: Young microspore (YM) with degenerated wall layers and well developed endothecial thickenings (ET); E and F: Anther locule with well stained healthy pollen grains (arrow) with distinguished exine and intine (arrows) (scale bar 0.1 mm).

In normal plants, the mature anther exhibited compressed epidermal cells, radially elongated endothecial cells with secondary wall thickenings (ET; Fig. 3E). The microsporangia were packed with mature pollen grains and there were no traces of tapetum (Fig. 3E). The pollen grains were dark stained confirming dense cytoplasmic contents with prominent exine and intine, indicating developed, healthy and fertile pollen grains (Fig. 3F). The stressed anthers also showed somewhat transparent epidermal cells and endothecial layer with secondary wall thickenings (ET; Fig. 4E). The stressed plants showed a low concentration of pollen grains in the anther locule (Fig. 4E) and in many cases they were irregular in outline and cytoplasm appeared to be almost absent (Fig. 4F). On the whole, due to lack of nourishment the pollen development appeared to be severely affected in plants grown under stress conditions compared to the control plants.

The whole mounts of the young microscopic buds also confirmed the above explained findings in young microspore development. Tetrads arrangement in both the conditions did not show any noticeable differences (Fig. 5A, B). Young microspores were observed with dense cytoplasmic content and prominent nucleus in unstressed plants (Fig. 5C; arrow). But in stressed ones during separation of the microspores out of tetrads, some of the pollen grains showed some abnormalities. Many young microspores had less cytoplasmic content and instead of being circular/spherical in outline, these were ovoid in shape (Fig. 5D, E).

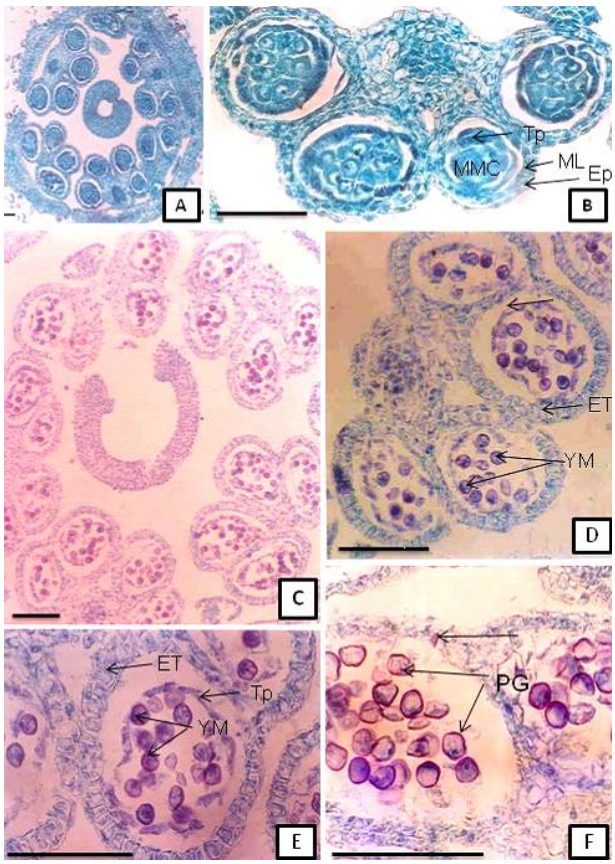

Figure 4. Transverse section (TS) of young microscopic bud of stressed (cold) plants showing different stages of microsporogenesis – A and B: TS of young bud showing tetrasporangiate anther at microspore mother cell (MMC) stage, with different wall layers (EP = epidermis, ML = middle layer, Tp = tapetum) enclosing MMC; C: Young bud showing young microspore with partial degenerated tapetal cells; D: Magnified view of a tetrasporangiate anther showing young microspore (YM) with partial degenerated tapetal cells (arrow); E: Further magnified view of anther locule showing less number of YMs, partially stained/transparent with developed endothecial thickenings (ET), partially degenerated Tp; F: Infertile pollen grains (PG) without cytoplasmic content and undeveloped ET (scale bar 0.1 mm).

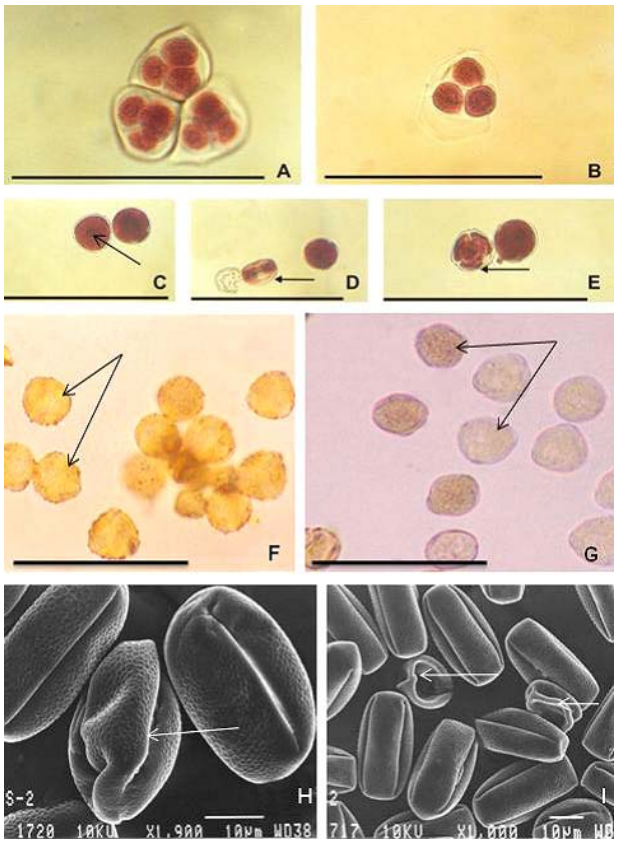

Figure 5. Whole mount preparations showing pollen development – A: Tetrads of stressed plant; B: Tetrads of unstressed plant; C: Unstressed young microspores with prominent nucleus (arrow); D and E: Stressed, undeveloped young microspore (arrows); F: Unstained pollen grains of control plants (arrow); G: Unstained pollen grains of stressed plants (arrow); H and I: Scanning electron micrographs of pollen grains with deformed shape (arrow) and shriveled/desiccated pollen grains in stressed plants (arrow) (scale bar 0.1 mm).

Pollen viability. Pollen viability in control plants was normal (91.3%) and the pollen grains were uniform in shape and size with dense cytoplasmic content; unstained pollen grains were yellow in color (Table 2). The shape of pollen grains varied from large, spherical and triangular (Fig. 5F; arrow). The stressed pollen grains were transparent, small sized, spherical or oval type (Fig. 5G; arrow). This difference in shape was also observed in preparations under the SEM. The scanning electron microscopy observations also indicated shrunken or desiccated pollen grains (Fig. 5H, I; arrows). Pollen viability was suppressed during stressed conditions in the present case. Apart from desiccated pollen the proportion of viable pollen was recorded as 53.1% in the stressed plants. This shrinkage or desiccation in pollen grains can be related to underdevelopment of pollen grains due to low temperature. Under dry conditions pollen grains with a distinct germination groove can collapse in this slit, and then get a more oval shape. Dry pollen has an angular or dented or sometimes even collapsed shape.

Stigma receptivity. Unstressed stigmas along with pollen were found to be normal as they developed reddish brown color (score 4; Table 2) as a result of esterase activity (Fig. 6A), while the stigmas of stressed plants exhibited lesser intensity of color (score 2) (Fig. 6C). Simultaneously freshly excised stigmas were gently pressed and observed for in vivo pollen germination. Control stigmas showed normal pollen germination (Fig. 6B), whereas stressed plants though possessed some pollen load showed no pollen germination (Fig. 6D).

Table 2. Comparison of flower functioning under warm and cold conditions (Mean ± SEM).

Parameter	Control (warm)	Cold-stressed
Pollen viability (%)	91.3 ± 2.7	53.1 ± 1.9
Pollen germination (%)	89.8 ± 2.3	42.6 ± 1.8
Pollen tube growth (µm)	19.4 ± 1.3	8.6 ± 1.3
Stigma receptivity (1–5 scale; esterase test)	4.0 ± 0.6	2.0 ± 0.43

Pollen germination and pollen tube growth. The gynoecia of control as well as stressed plants were examined from day of anthesis to investigate the pollen load, pollen germination and finally to trace the pollen tube growth in the stigma, style and ovary. In chickpea, self-pollination occurs one to two days before anthesis and fertilization occurs 24 h after pollination, so flowers were examined from day of anthesis to 3 days after anthesis. In plants growing under control conditions, a large number of pollen grains, pollen germination (89.8%; Table 2) and pollen tubes (19.4 µm; Table 2) could be seen growing through the stigma and style (Fig. 7A, B) and eventually reaching the micropylar region of the ovule (Fig. 7C, D). On the other hand, in plants grown under stressed/field conditions, different situations were observed. In stressed plants, the stigma either did not show any pollen load (Fig. 8A; arrow) or pollen grains showed poor germination (Table 2). The pollen tubes of germinated pollen rarely grew beyond the proximal region of the style (Fig. 8B; arrow) and had reduced length (8.6 µm; Table 2). Mostly, the pollen tubes did not reach the ovules (Fig. 8C) which led to failure in fertilization. In rare cases, very less numbers of pollen tubes were seen reaching the ovary (Fig. 8D; arrow).

Post-pollination and fertilization changes. Relatively, female components were affected to a lesser extent than male. We focused mainly on post-pollination and fertilization events. In longitudinal sections of young

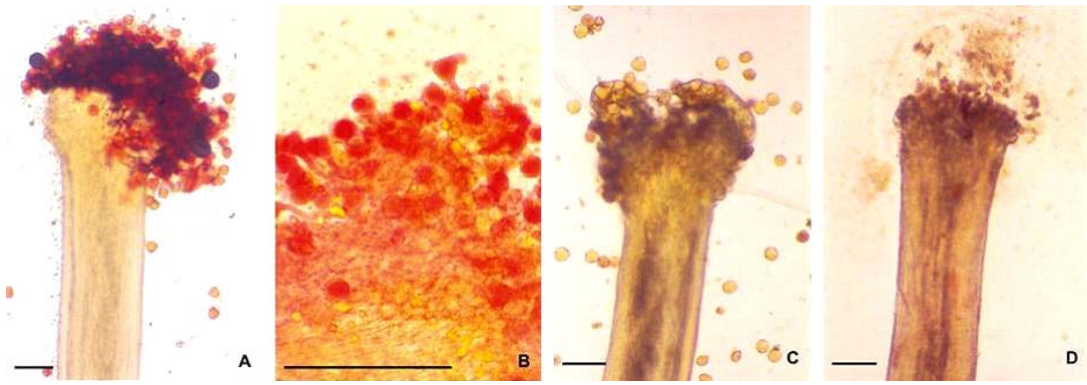

Figure 6. Stigma receptivity and in vivo pollen germination – A: Stigma receptivity and pollen load in unstressed plant; B: In vivo pollen germination in unstressed plant; C: Stigma receptivity in stressed plant; D: In vivo pollen germination in stressed plant (scale bar 0.1 mm).

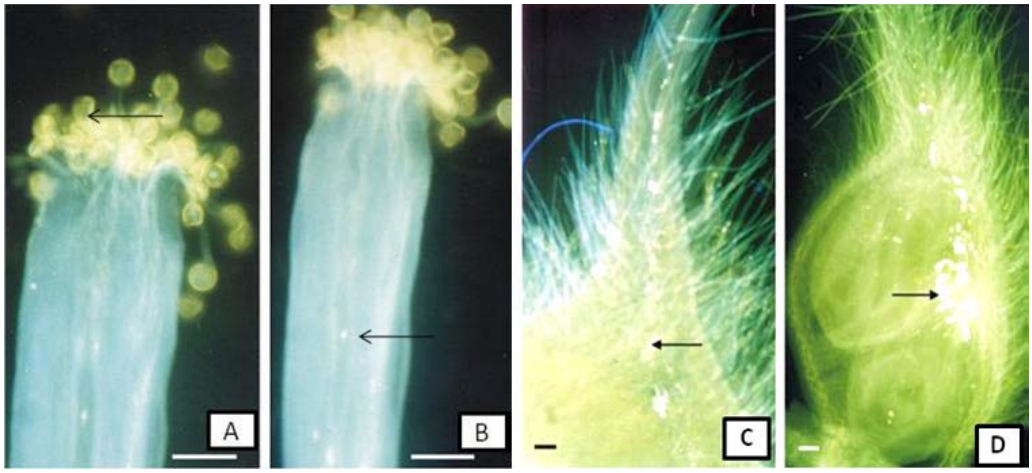

Figure 7. Pollen germination and pollen tube growth studies in the flowers of control plants – A and B: Germinating pollen grains on the stigma (arrows); C: Pollen tubes traveling in the stylar region (arrow); D: Pollen tubes entering the ovary and ovules (arrow) (scale bar 0.1 mm).

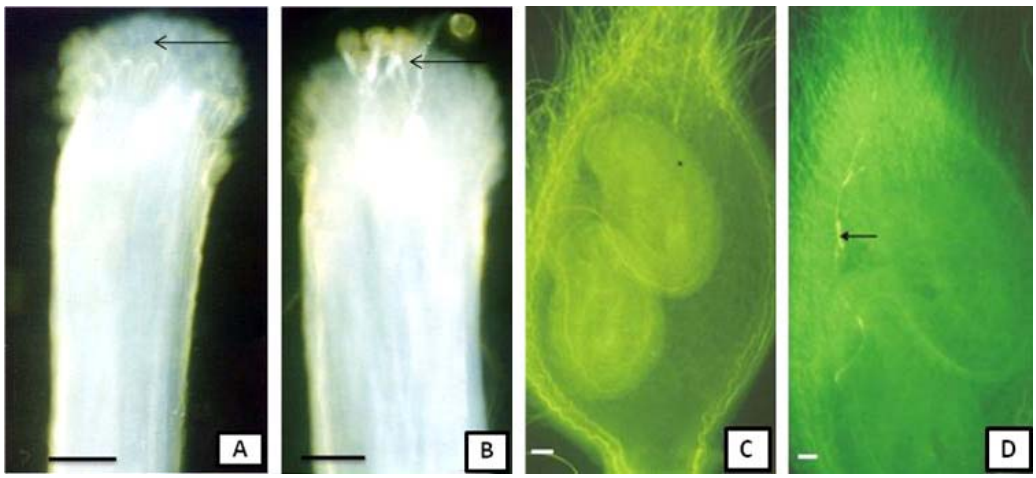

Figure 8. Pollen germination and pollen tube growth studies in the flowers of stressed plants – A: No pollen load on stigmatic surface (arrow); B: Pollen load and germination on stigmatic surface (arrow); C: No pollen tubes entering the ovary and ovule; D: Very less pollen tubes entering the ovary and ovule (arrow) (Observations taken three days after anthesis; scale bar 0.1 mm).

seeds, under unstressed conditions, the embryo sac showed a zygote (Z; Fig. 9A, B) and normal development of pro-embryo (Pro; Fig. 9C) as well as endosperm nuclei (EsN; Fig. 9D). In the stressed plants, the embryo sac soon after anthesis showed an egg, but there was no indication of syngamy and triple fusion. The egg and secondary nucleus in this ovule ultimately disintegrated and hence there was no seed formation (Fig. 9E, F; arrows). The egg apparatus also remained functional for some time and eventually degenerated before the pollen tube entry into the ovules. The embryo sac along with the surrounding integuments increased in size for some time but eventually the ovule aborted.

Discussion

The accumulation of anthocyanins in cold-stressed chickpea plants appears to be associated with the low temperature effect because no such pigmentation was observed in control conditions. The underlying reasons of accumulation of these pigments in response to cold stress are not known but evidently being an antioxidant, it may have a role in stress protection (Chalker-Scott 1999). Its accumulation in the present case suggests its use as a potential indicator of cold sensitivity in chickpea. Anthocyanins are reported to accumulate in response to cold stress in other plant species too (Janda et al. 1996, Hasegawa et al. 2001).

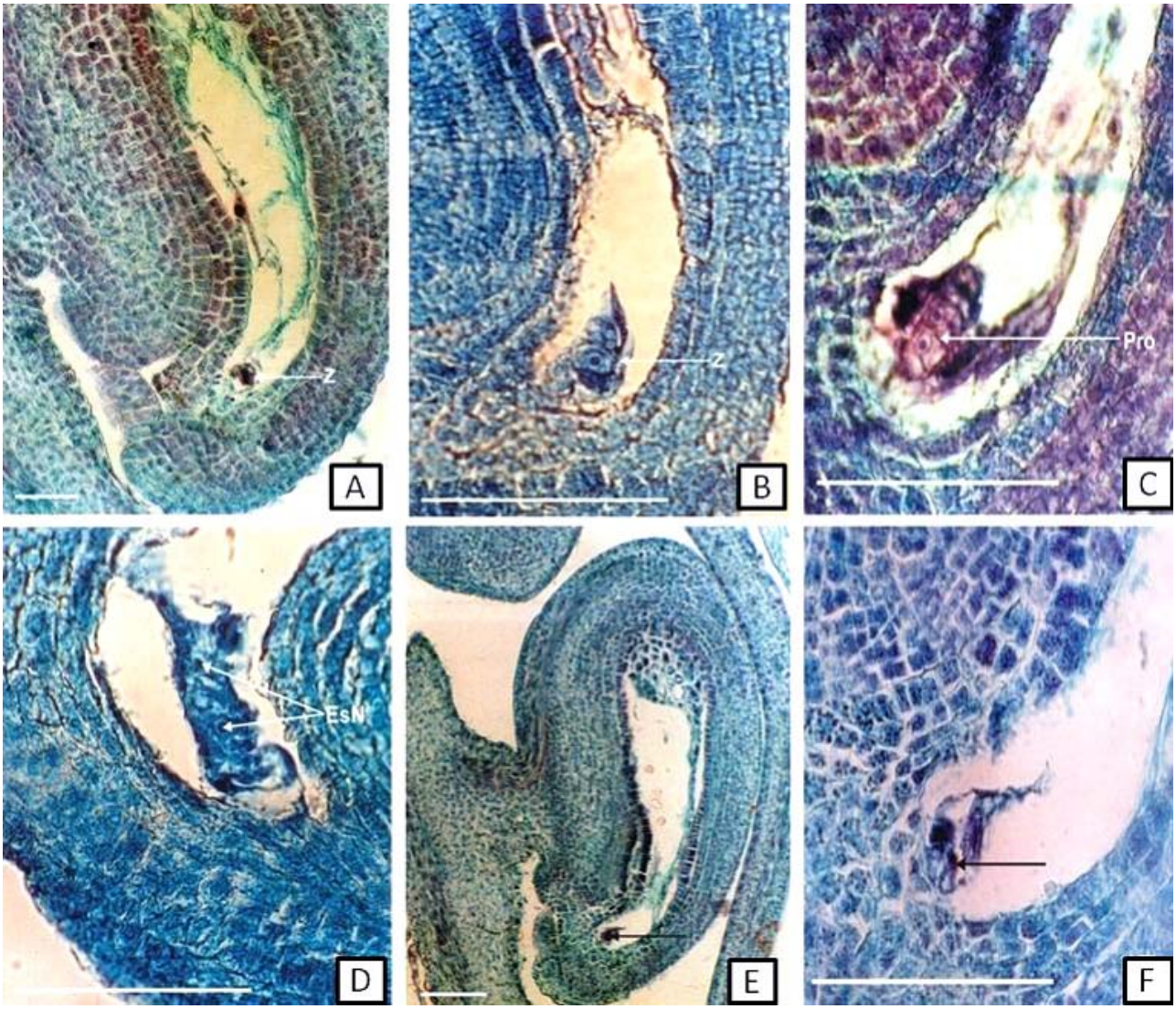

Figure 9. Longitudinal section (LS) of young seed (ovule) showing fertilization in unstressed plant (A to D) and stressed plant (E and F) – A and B: Zygote (Z) formation; C: Pro-embryo (Pro) stage; D: Endosperm nuclei (EsN); E: LS of ovule showing degenerated egg apparatus in the micropylar region (arrow) of the aborting flower of stressed plant; F: Magnified view of E showing degenerated egg apparatus in the micropylar region (arrow) (scale bar 0.1 mm).

The field-grown plants showed more abscission of flowers (66%), less floral retention (33.6%) and pod set (35.5%) than glasshouse plants (39%, 60.5%, 44.4%, respectively). In chickpea, floral abscission is the most common damage caused by low temperature (<10°C) and has been reported in earlier studies (Srinivasan et al. 1998, 1999, Clarke and Siddique 2004, Kumar et al. 2005, Nayyar et al. 2005, Berger 2007). Cold stress results in floral abscission in other legumes also, such as soybean (Gass et al. 1996) and pigeonpea (Sandhu et al. 2007). In soybean, severe chilling causes 100% flower abortion which results in plants without pods (Saito et al. 1970, Schori et al. 1993). In an earlier study (Nayyar et al. 2005), we reported that floral abnormalities might arise due to marked elevation of abscisic acid content in flowers destined for abortion. This was suggested to inhibit the sucrose uptake into these flowers leading to their abortion.

Low temperature in our studies appears to affect both the structural as well as functional aspects of the male and female components of the chickpea flowers. The functioning of male and female components was observed to be inhibited to a different extent in our studies, which is in agreement with the findings of Srinivasan et al. (1999) and Croser et al. (2003). Regarding pollen development, we observed that after their separation from tetrads, some

of the young microspores were ovoid or spherical in shape and had very less cytoplasmic content suggesting impairment in their development.

The healthy development of young microspore depends upon anther wall layers, their functioning, degeneration and further development into secondary wall thickenings. Any abnormality at one or the other stage due to environmental aberrations may impair the pollen development. Tapetum layer is one such tissue which plays an important role in the development of pollen grains and pollen fertility. In our study, we have observed delayed degeneration of the tapetum especially in the cold-stressed anthers, and the production of non-fertile pollen grains may be a consequence of this event. Satake and Hayase (1970) in rice also reported that the period of greatest chilling sensitivity during anther development was the early microspore phase, during which tetrads develop into early microspores and during this phase if the plant undergoes cold stress, the anthers become smaller and the surrounding tapetal cells which provide nutrition to the developing microspores undergo hypertrophy followed by breakdown. Consequently, the normal development of pollen grains does not occur and the pollen grains at maturity are functionally sterile and contain very little or no starch. Nishiyama (1976) also observed alterations at the cellular level during microspore development under cold stress in rice. It was observed that most of the changes in tapetal morphology due to cold stress were found to be associated with dilatation called tapetal hypertrophy. On the other hand, Imin et al. (2004) observed enhancement of partial degradation of tapetal proteins after cold treatment, demonstrating for the first time that low temperature stress at the young microspore stage enhanced and induced partial degradation of proteins in the rice anthers at the trinucleate stage. In rice, Oliver et al. (2005) demonstrated that cold sensitivity was associated with the period when tapetum activity was highest. A gene encoding for cell wall bound invertase in tapetum was reported to be suppressed by cold stress experienced at young microspore stage.

A reduction in the viability of pollen grains in cold-stressed plants in comparison to the normal ones was observed here. The difference in size and shape was also noticeable under contrasting conditions. This shrinkage or desiccation in pollen grains can be related to under-development of pollen grains due to low temperature. In earlier such studies too, pollen viability was reported to decrease in chickpea (Srinivasan et al. 1999, Kumar et al. 2005, Nayyar et al. 2005) as well as in other plant species affected by cold stress (Demotes-Mainard et al. 1995, Mercado et al. 1997, Choudhury et al. 2002). Alterations in pollen shape might be the result of impairment in their development due to low temperatures as observed previously in maize (*Zea mays*) (Frascaroli 1995), wheat (*Triticum aestivum*) (Demotes-Mainard et al. 1995) and pepper (Mercado et al. 1997). In mango (*Mangifera indica*), cold periods are associated with reductions in fruit set and it was demonstrated that night temperatures below 10°C decreased pollen viability to 50% of controls (Issarakraisila and Considine 1994). Earlier, we reported pollen of aborted flowers possessed more abscisic acid and less sucrose content than those of retained flowers in chickpea (Nayyar et al. 2005). Thus, reduced pollen viability observed here might be related to impairment in the nutritive tissue, which leads to underdeveloped pollen grains having no reserves.

Pollen tube growth was retarded under in vivo conditions, which might occur due to direct chilling effects on the tube growth or because of starvation of tube for nutrients due to limitations in their supply to female components. It was suggested earlier (Nayyar et al. 2005) that the style of cold-stressed chickpea flowers had relatively small amount of sucrose than the unstressed ones indicating a restriction in transport of assimilates.

Stigma receptivity was considerably less in cold-stressed plants. Furthermore, in vivo pollen germination on stigma of the stressed plants was absent compared to control plants. Pollen tubes growing in vitro are known to take up carbohydrates and amino acids (Kendall et al. 1971) and presumably, these and other nutrients are supplied by the style under in vivo conditions. The reduced pollen germination and slow or distorted pollen tube growth on the stigma could be due to the low amounts of exudates on the stigma as well as decreased supply of reserves by the female components due to stress. This indicated that the female components were functionally inhibited by cold stress to suppress the congenial environment for pollen germination and pollen tube growth. Additionally the external temperature may inhibit pollen germination and also restrict the pollen tube which remains in the mid way or does not cross the proximal region of the style. Consequently the egg apparatus awaiting pollen tube degenerates. Therefore, apart from the developmental aberrations in male and female components, their interaction is also affected by cold which leads to failure in fertilization and/or no pod set.

The effects of chilling temperatures on the development of female gametophyte, ie, embryo sac has so far received very less documentation. The impairment in the embryo sac development influenced by cold stress may occur due to damaged process of megasporogenesis or megagametogenesis. Srinivasan et al. (1998) demonstrated that low temperature reduced the size of ovary in chickpea. Their results also showed that the number of ovules formed was unaffected by cold stress; however, ovule size and viability was reduced in all

cultivars. The reduction in ovule viability due to low temperature stress in chickpea was also documented by Nayyar et al. (2005). Westgate and Boyer (1985) stated that ovule sensitivity to stress starts at anthesis and early stages of grain development. Lardon and Triboi-Blondel (1994) described that ovule sensitivity to cold stress in winter rapeseed (*Brassica napus*) increases up to anthesis. They showed that at the stage when ovules became sensitive to cold they were inside the buds and meiosis had just initiated, but maximum sensitivity was observed when the flower was nearing anthesis. About 80% ovules were found injured at that stage. Casper (1990) reported irreparable ovule abortion in *Cryptantha flava* under cold stress conditions due to failure of fertilization in cold sensitive cultivars.

A complete perusal leads us to the conclusion that the development of male reproductive parts at early microspore stage was affected by cold stress, which gives rise to malnourished pollen grains that are impaired in their germination. Poor stigma receptivity restricted the pollen germination and pollen tube growth was also repressed in the style leading to fertilization failure. Additionally, the development of egg as well as embryo was also abnormal contributing towards the impaired fertilization. Future studies require to be focused on nutritional aspects of floral development in relation to cold stress, which may provide better clues about floral abortion.

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