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Effect of high temperature on the reproductive development of chickpea genotypes under controlled environments

Viola Devasirvatham[1, 2]*, Pooran M Gaur [2], Nalini Mallikarjuna[2], Raju N. Tokachichu[1], Richard M. Trethowan [1], and Daniel K.Y. Tan [1]

[1]Faculty of Agriculture and Environment, Plant Breeding Institute, University of Sydney, Cobbitty, NSW 2570. [2]International Crops Research Institute for the Semi-Arid Tropics, Patancheru, Hyderabad, 502324, A.P., India.

Corresponding author: [1, 2]*Suite 401, Biomedical Building, 1 Central Avenue; Australian Technology Park, Eveleigh, NSW, Australia, 2015. Phone: +61 2 8627 1175; Fax: +61 2 8627 1099; Email: viola.devasirvatham@sydney.edu.au*; p.gaur@cgiar.org; n.mallikarjuna@cgiar.org; Tokachichu.raju@sydney.edu.au; Richard.Trethowan@sydney.edu.au; Daniel.Tan@sydney.edu.au

Running Title: High temperature and reproductive development in chickpea

Abstract

High temperature during the reproductive stage in chickpea (*Cicer arietinum* L.) is a major cause of yield loss. The objective of this research was to determine if that variation can be explained by differences in anther and pollen development under heat stress. Therefore the effect of high temperature during the pre- and post-anthesis periods on pollen viability, pollen germination in a medium, pollen germination on the stigma, pollen tube growth and pod set in a heat tolerant (ICCV

92944) and a heat sensitive (ICC 5912) genotype was studied. The plants were evaluated under heat stress and non-heat stress conditions in controlled environments. High temperature stress (29/16˚C to 40/25˚C) was gradually applied at flowering to study pollen viability and stigma receptivity including flower production, pod set and seed number. This was compared with a non-stress treatment (27/16˚C). The high temperatures reduced pod set by reducing pollen viability and pollen production per flower. The ICCV 92944 pollen was viable at 35/20˚C (41% fertile) and at 40/25˚C (13% fertile), while ICC 5912 pollen was completely sterile at 35/20˚C with no *in vitro* germination and no germination on the stigma. However, the stigma of ICC 5912 remained receptive at 35/20˚C and non-stressed pollen (27/16˚C) germinated on it during reciprocal crossing. These data indicate that pollen grains were more sensitive to high temperature than the stigma in chickpea. High temperature also reduced pollen production per flower, % pollen germination, pod set and seed number.

Keywords

Anther, high temperature, pollen viability, pre-anthesis, post-anthesis

Introduction

Whilst chickpea (*Cicer arietinum* L.) is considered to be a cool season legume, it is often grown where temperature exceeds >30˚C during the reproductive stage, which can limit yield (Summerfield *et al*. 1990). The negative effect of high temperature on grain yield is expected to increase due to global warming. A minimum decrease of 53 kg/ha of chickpea yield was observed in India per 1˚C increase in seasonal temperature (Kalra *et al*. 2008).

Heat stress during reproductive development in legumes is generally allied with lack of pollination and abscission of flower buds, flowers and pods, leading to substantial yield loss (Nakano *et al*. 1997, 1998; Duthion and Pigeaire 1991). For example, short periods (10 day) of high temperatures (≥ 35˚C) during early flowering and pod development of chickpea are known to cause significant reduction in pod number, seed set and grain yield (Wang *et al*. 2006). Grain yield reduction was due to high temperature effects on pre-anthesis, post-anthesis development and pollination. Nayyar *et al*. (2005) suggested that the male (pollen, anthers) and female (stigma-style, ovary) organs in chickpea are most sensitive to temperature stress. Therefore the period of anthesis and seed set may be critical with respect to high temperature tolerance (Summerfield and Wien 1980; Gross and Kigel 1994).

Male reproductive development and fertility are sensitive to heat (Sakata and Higashitani 2008). Anthers can be influenced by high temperature. Anther indehiscence occurs in cowpea (*Vigna unguiculata* L.) due to heat stress (33/30˚C) and is associated with degeneration of tapetal layer (Ahmed *et al*. 1992). The degeneration of tapetum cells was also found in common bean (*Phaseolus vulgaris* L.) at 33/29˚C (Suzuki *et al*. 2001), resulting in premature pollen development within the anther during early development. High temperature (33/27˚C) before anthesis can also cause anther indehiscence and pollen sterility in common beans (Gross and Kigel, 1994). In legumes, reductions in pollen production and fertility, and increases in pollen abnormalities (small, shrunken and empty pollen grains) occur during pre-anthesis at high temperatures. Warm night temperatures (28˚C) reduce pollen production in groundnut (*Arachic hypogaea* L.) (Prasad *et al*. 1999b) and associated with yield loss (Prasad *et al*. 1999a). Chickpea genotype ICC 5912 became sterile showing heat sensitivity with exposure at 35/20˚C a day before anthesis (1 DBA), but the pollen of chickpea genotype ICCV 92944 pollen was fertile at the same temperature (Devasirvatham *et al*. 2010). Halterlein *et al*. (1980) reported that pollen viability in common bean decreased when temperatures were held at 35/20˚C or 35˚C for a 24 h period just before anthesis. Shrunken pollen was observed at 38/30˚C in heat tolerant soybean (*Glycine max* L.) (Koti *et al*. 2005).

In legumes, post-anthesis high temperature effects are associated with poor pollen germination on the stigma and reduced pollen tube growth in the style (Talwar and Yanagihara, 1999), failure of fertilisation (Ormrod *et al*. 1967) and embryo abortion (Gross and Kigel, 1994). Chickpea pods will generally form 5-6 days after pollination in the field (Singh and Diwakar 1995), and on the 5th day in controlled environments (Bassiri *et al*. 1987). Generally the peak grain filling period is 20 days after end of flowering (Ozalkan *et al*. 2010) but varies with genotype and environment. However there is no evidence of parthenocarpic pod formation under abiotic stress in chickpea. Chickpea genotypes differ in response to heat based on physiological (photosynthesis and membrane stability) and biochemical (enzyme) mechanisms. For example membrane stability in chickpea was higher (> 40˚C) than pollen viability (≥ 35˚C) (Basu *et al*. 2009) at high temperature. To date, there is no published data linking these mechanisms to reproductive stage tolerance such as pollen viability, pod set, seed set and grain yield. Whilst under cool conditions the mechanism of pollen development and fertilisation in chickpea has been elucidated (Srinivasan *et al*. 1999; Clarke *et al*. 2004), it is not well understood at high temperatures.

Heat tolerant genotypes of chickpea were identified from field screening in India (Krishnamurthy *et al*. 2011; Upadhyaya *et al*. 2011), and heat tolerance in ICCV 92944 has been observed under field conditions (Gaur *et al*. 2010). Therefore ICCV 92944 was selected as a heat tolerant source and ICC 5912 as a relatively sensitive genotype. The development of pollen viability screens for high temperature stress has provided a useful tool for breeding temperature tolerant chickpea varieties. The aim of this research was to determine if differences in high temperature effects on pollen viability, pollen germination, pollen tube growth and pod set can explain the relative heat tolerance/ sensitivity of ICCV 92944 and ICC 5912 chickpea genotypes.

Materials and methods

Two controlled environment experiments were conducted with two chickpea genotypes (ICCV 92944 and ICC 5912) at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), (17.53^{o}N; 78.27^{o}E; 545 m), Hyderabad, India in 2010 and 2011. Plants of the two genotypes were grown in a controlled environment with five replications. Each replication has five plants i.e. one plant per pot. Three seeds of each genotype were sown in pots (2.4 L volume) containing a mixture of black vertisol soil, sand and vermi-compost (4:2:1 by volume), later seedlings were thinned to one per pot. Because the phenology of the two genotypes differs, they were sown on different dates to synchronise anthesis. The plants were grown at 27/16˚C in a greenhouse and transferred to a growth room to expose them to high temperature at the first appearance of flowers. The plants used as a non-stressed control continued to grow in the glasshouse at 27/16˚C under natural light. The temperature in the growth room was increased daily by 1˚C, e.g. 28 to 40˚C during the day and 16 to 25˚C during night (Table 1) in a square wave form. Therefore, the plants were exposed to a gradual increase in temperature to identify the critical temperature above which reproductive development begins to fail. The temperature was constantly maintained in the growth chamber with a 15 min transition period from day to night temperature and vice versa. The growth room had 72% input wattage of 1500-mA cool white fluorescent and 28% input wattage of Sylvania 50W-277V incandescent lighting. The light intensity (quantum) was about 320 µmol s^{-1} m^{-2} (Light meter model LI-189 from Li-Cor; USA) during 12 h photoperiod (08 to 20 h) and relative humidity was 75-80% in the growth chamber. Careful watering ensured that moisture was not a limiting factor in either temperature regime (day/night).

Experiment-1

The effects of a one day exposure to day/night temperatures ranging from 31/16˚C to 40/25˚C one day before anthesis (termed pre-anthesis period) were studied to determine the critical temperature for pollen viability. Five flower buds were collected between 08:00 and 08:15 h from 31/16˚C to 40/25˚C to examine pollen viability during pre-anthesis. Non-dehiscent anthers were stained with Alexander's stain procedure-3 (Alexander, 1969). The samples were examined under a compound microscope (Figs. 1a to 1e). The fertile pollen grains inside the anthers were red in colour whilst the sterile pollen grains were green. The differentiation of fertile and sterile pollen grain was found to depend on the pollen wall thickness and the chemical composition and pH of the stain. Malachite green was used to stain the pollen grain wall. Therefore, sterile pollen grains appeared green in colour. The protoplasm in the pollen grain was stained by acid fusion used in the Alexander's stain and hence it coloured the fertile pollen grain red to deep red (Alexander, 1969). All open flowers in the high temperature treatments (32/17˚C to 40/25˚C) were tagged to observe the pod set. Pod formation and withered pods were recorded on 7th day from tagging of the open flower.

ICCV 92944 was examined at 40/25˚C (extreme temperature) for pollen viability. At 40/25˚C, the pollen viability in all 10 anthers of the heat tolerant genotype was observed using 2% acetocarmine stain and replicated three times. Each anther was squashed and mounted on a slide. Stained (fertile) and non-stained (sterile) pollen grains were counted and percentage of pollen fertility was determined.

In vitro pollen germination and tube growth were assessed using pollen germination medium (Mallikarjuna *et al*. 2007). Pollen germination (i.e. fertile and sterile pollen per flower) was counted at 35/20˚C and compared with the control at 27/16˚C. Two flowers per temperature treatment were collected the next morning between 08:00 and 08:15 h and each flower considered a replication. The available pollen grains in a flower were carefully transferred to the slide. The number of pollen grains (germinated and non-germinated) was counted in horizontal microscopic observation field using all pollen grains in the microscope field. Fifty such observations were made per replication in high temperature treatments. One hundred observations were made per replication (flower) in the control treatments. Therefore the average from the 50 or 100 observations per flower was considered to be a replication. The pollen production per flower was determined by summing the fertile (germinated) and sterile (non-germinated) pollen grains.

To evaluate the effect of different temperature regimes (33/18˚C to 40/25˚C) on pollen viability, the percentage of pollen germination was calculated. Under the high temperature treatment in pollen grains, the flower buds (one day before anthesis) were exposed from 33/18˚C to 38/23˚C and the flowers were collected the next day morning between 08:00 and 08:15 h and *in vitro* pollen germination and pollen tube growth determined. Two flowers per temperature treatment were collected and each flower considered a replication. The available pollen grains in a flower were carefully transferred to the slide. The *in vitro* pollen germination was terminated after 60 min incubation by adding a drop of 2% acetocarmine stain. Pollen grains were counted as germinated when pollen tubes were at least equal to the diameter of the pollen grain by the random microscopic field observation method. Percent pollen germination was determined by using all pollen grains in a microscope field as a pseudo replication; 15 such observations were made. The average from the 15 observations per flower was considered to be a replication. Therefore two replications per temperature treatment were used to calculate pollen germination.

Experiment-2

Similar to experiment-1 the temperature was increased daily by 1˚C, e.g. 28 to 35˚C during the day and 16 to 20˚C during night (Table 1). Hand pollination was carried out between 08:00 and 08:30 h at 35/20˚C and 27/16˚C to examine *in vivo* pollen germination and tube growth. Stressed stigma x stressed pollen and stressed stigma x non-stressed pollen crossing was done in the growth chamber. Non-stressed stigma x stressed pollen crossing was done in the glasshouse.

Reciprocal crosses (stressed x non-stressed; non-stressed x stressed) were carried out to determine the site of sensitivity; whether it was the pollen or the stigma responsible for high temperature stress susceptibility. The following crosses were made

i. Stressed stigma (35/20˚C) x stressed pollen (35/20˚C) within the same genotypes
ii. Stressed stigma (35/20˚C) x stressed pollen (35/20˚C) between the genotypes
iii. Non-stressed stigma (27/16˚C) x stressed pollen (35/20˚C) between the genotypes
iv. Non-stressed stigma (27/16˚C) x stressed pollen (35/20˚C) within the same genotypes
v. Stressed stigma (35/20˚C) x non-stressed pollen (27/16˚C) within the same genotypes

Seven flowers were pollinated per one crossing combination and each flower was considered a replication. Each stigma (flower) was pollinated with pollen grains from one flower. Therefore seven flowers per one crossing combination were pollinated with seven different flowers from the male parent (i.e. different plants). The flower samples were collected 15 min and 30 min after pollination to observe pollen germination on the stigma and pollen tube growth down the style. The flowers were fixed for 24 h in 80% alcohol. The pistils (styles and ovary) were removed from the flowers, cleared with 6 N NaOH for 48 h and thoroughly rinsed with water. The pistils were stained with aniline blue and observed under a fluorescence microscope.

Statistical analysis

Analysis of variance (ANOVA) was performed for flower and anther data, pollen germination count and percentage of different temperature regimes using Genstat 12th Ed. VSN International Ltd. One way ANOVA was conducted at for ICCV 92944 at 40/25˚C for all 10 anthers with three replications. Two way ANOVA (genotype × temperature) was conducted with two replications for pollen production per flower and pollen germination (%). For floral morphology (%) and pod characters, two way ANOVA (genotype × temperature) was performed with five replications to study the difference between heat stressed and non-stressed conditions.

Results

Pollen fertility studies using Alexander and Acetocarmine stains

The one day exposure of flower buds to high temperature influenced pollen fertility. In ICC 5912, the pollen grains inside the anthers were fertile up to 34/19˚C (red colour pollen grains) (Fig. 1a). However, at 34/19˚C, anthers showed abnormalities (Fig. 1a; supplement Fig. 1). Increased numbers of locules in the anther indicated heat damage. At 34/19˚C, the sensitive genotype ICC 5912 had more than three locules (Fig. 1a). High temperature affected the pollen sterility (Figs. 1a, e). The anthers contained a mixture of fertile and sterile pollen grains (partial sterility) and viable pollen within the anther was found at 34/19˚C (Fig. 1a). However, at 35/20˚C, pollen grains inside the anther of ICC 5912 became sterile (Fig. 1b). The sterile pollen grains were uniformly distributed in all 10 anthers of a flower in ICC 5912 at 35/20˚C. At 40/25˚C, the anthers were devoid of pollen grains (supplement Fig. 1e). In addition, there was no pod set in ICC 5912 at 35/20˚C, indicating that this is the critical temperature for cessation of pod set in this genotype.

Pollen grains of ICCV 92944 remained fertile at 36/21˚C (supplement Fig. 1). Though ICCV 92944 had reduced the pod set, pollen fertility was observed up to 38/23˚C (Fig. 1d). However, at 37/22˚C, few anthers showed indehiscence due to anther wall thickness (Fig. 1c). At 40/25˚C, the pollen grains inside the anther were partially sterile in ICCV 92944 as observed with Alexander's stain (Fig. 1e). Among 10 anthers observed, two were completely sterile (Table 2). The sterility in the remaining eight anthers ranged from 85 to 97%. The temperature rise from 35/20˚C to 40/25˚C led to complete pollen grain sterility (P<0.05). ICCV 92944 ceased to flower, moved into a pod development phase and showed symptoms of leaf yellowing (maturity), while the heat sensitive genotype ICC 5912 retained green leaves and continued to flower without pod setting.

In vitro pollen germination and tube growth

High temperature (35/20˚C) reduced pollen production and pollen fertility (Tables 3 and 4). Generally at high temperature (38/23˚C) the sterile pollen was high (91%) (Table 4). In ICCV 92944, pollen germination (%) and pollen production at 35/20˚C was reduced by more than 50% compared with the optimum temperature (27/16˚C). At 35/20˚C in ICC 5912, the percentage of pollen germination was zero; only sterile pollen was produced (Table 4). Therefore high temperature influenced pollen fertility. At 34/19˚C, ICC 5912 had both normal (straight, smooth and cylindrical) (supplemental Fig 1f) and stressed/ abnormal (zigzag) tubes (supplemental Fig. 1g), which suggested inhibition of pollen tube growth at high temperature. Pollen germination within the anther may be a sign of heat stress (Fig. 1f) and was observed before reaching the critical temperature (35/20˚C). 39% sterile pollen of ICC 5912 was found at 34/19˚C (Table 4). The pollen germination (%) in ICCV 92944 declined from 64% at 33/18˚C to 19% at 38/23˚C (P<0.001) (Table 4). Overall pollen germination was 9% at 38/23˚C (Table 4). However, length of pollen tube growth varied from 35/20˚C (supplemental Fig. 1h) and bursting of pollen grains including release of protoplasm occurred at 38/23˚C (Fig. 1i). Therefore the high temperature of 38/23˚C was not conducive for pollen tube growth.

Flower production and pod set in different temperature regimes

Flower formation was reduced at high temperature compared with optimum temperature with the number of flowers reduced (P<0.001) from 10 (27/16˚C) to three (38/23˚C). ICC 5912 did not set pods at 35/20˚C. The seed set % was also zero in ICC 5912 at 35/20˚C. In contrast, ICCV 92944 continued to set pods up to 38/23˚C, but % pod set in ICCV 92944 was reduced along with flower formation. Therefore the seed was low in ICCV 92944 at high temperature.

Pod characters and plant biomass under high temperature

At high temperature stress, number of pods/plant, filled pods/plant and seeds/plant was reduced (P<0.001) from 41, 37, 37 (27/16˚C) to 15, 11 and 13 (heat stress) respectively (Table 6). Despite seeds being formed at high temperature stress, most of the filled pods or seeds were formed before the temperature reached 35/20˚C. Generally, the flower production was reduced after 36/21˚C. The pollen grains of ICC 5912 were fertile at 35/20˚C. The plant biomass was also reduced from 15.3 (27/16˚C) to 10.9 g/plant under heat stress. Therefore high temperature reduced grain yield and biomass accumulation.

In vivo pollen germination and tube growth

i. Stressed stigma (35/20˚C) x stressed pollen (35/20˚C) within the same genotype

Pollen germination and pollen tube growth was found in ICCV 92944 at 35/20˚C (Fig. 1j). Though the pollen tube had callose[1] formation, the tube reached the base of the style at 30 min (Fig. 1k). However there was no pollen germination on the stressed stigma of ICC 5912 at 35/20˚C.

ii. Stressed stigma (35/20˚C) x stress pollen (35/20˚C) between the genotypes

Stressed pollen from ICC 5912 did not germinate on the stigma of ICCV 92944 (Fig. 1l), but stressed pollen grains from ICCV 92944 germinated on the stressed stigma of ICC 5912 and formed uneven pollen tube growth after large callose formation under stress (35/20˚C) (Fig. 1m). After 30 min the callose deposition was reduced in the style.

iii. Non-stressed stigma (27/16˚C) x stressed pollen (35/20˚C) between the genotypes

The stressed pollen from ICC 5912 did not germinate on the non-stressed stigma (27/16˚C) of ICCV 92944 (Fig. 1n). However, the stressed pollen from ICCV 92944 germinated on the non-stressed stigma of ICC 5912 (Fig. 1o). After 30 min reduced callose formation was found in the non-stressed style (27/16˚C) of ICC 5912.

iv. Non-stressed stigma (27/16˚C) x stressed pollen (35/16˚C) within the same genotype

In ICCV 92944, the stressed pollen (35/20˚C) was germinated (Fig. 1p) on the non-stressed stigma (27/16˚C) with smooth tube growth 30 min after pollination. However, in ICC 5912 the stressed pollen (35/20˚C) did not germinate on its non-stressed stigma (27/16˚C) (Fig. 3r).

v. Stressed stigma (35/20˚C) x non-stressed pollen (27/16˚C) within the same genotype

In ICCV 92944, the non-stressed pollen (27/16˚C) was germinated on the stressed stigma (35/20˚C) (Fig. 3q). Similar germination was found on the non-stressed ICC 5912 pollen (Fig. 3s). The pollen tube growth was smooth with little callose deposition near the ovary (Fig. 3t).

Discussion

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[1] Callose, an amorphous, colourless substance, is a β-1, 3-polyglucan composed of β-D glucopyranose residues. It is insoluble in water and ethanol. Callose forms along the inner pollen tube membrane and restricts tube cytoplasm (Stanley and Linskens, 1974).

This study demonstrated that a heat-tolerant and a heat-sensitive genotype in chickpea varied in pollen viability before anthesis, pollen germination at anthesis, and pollen germination and pollen tube growth on the stigma when exposed to high temperature (day/night). High temperature caused three types of damage: an increase in locule number; partial sterility, and pollen germination within the anther. These were observed in the heat sensitive genotype ICC 5912 at 34/19˚C while partial sterility was the only damage observed in heat tolerant genotype ICCV 92944 at 40/25˚C. This study confirmed that pre-anthesis heat stress resulted in anthers with changed locule numbers in the sensitive genotype. In ICC 5912, the abnormal anther (Fig. 1a) was larger in size (length and width) than the normal anther (supplement Fig. 1a) at 34/19˚C because of increased numbers of locules. Normal anthers (supplement Fig. 1a) have two locules but the high temperature stressed anthers produced more than three locules (Fig. 1a, supplement Fig. 1a). This observation may reflect changes in anther development phase-1 (changes in anther cell differentiation) and anther development phase-2, especially changes in stomium which is responsible for anther dehiscence (Goldberg *et al*. 1993). Changes in locules number were also reported in cowpea at 33/30˚C (day/night) that was exposed to heat 5-7 DBA (Warrag and Hall 1984). Changes in anther shape and stomium opening at 32/27˚C (day/night) was found in a heat tolerant common bean genotype after 13 days of pre-anthesis heat treatment (Porch and Jahn, 2001).

The temperature sensitive genotype ICC 5912 showed a mixture of fertile and sterile pollen grains (partial sterility) in the anther at 34/19˚C, but only partial sterility was found in the heat tolerant genotype ICCV 92944 at 40/25˚C. Partial sterility (a mixture of fertile and sterile pollen grains) occurred in wheat (*Triticum aestivum* L.) at exposure to 30˚C (day/night) for three days pre-anthesis (Saini *et al*. 1984). Variation in pollen fertility occurred among the flowers and among the anthers within the flower in ICC 5912 at 34/19˚C and in ICCV 92944 at 40/25˚C (Table 2). The pollen germination within the anther before anthesis was observed without any artificial supplementary nutrients (e.g. sucrose) in the sensitive genotype at 34/19˚C. Generally, the Fabaceae family (e.g. chickpea) has 1-10 min lag period before pollen germination because the pollen is equipped with fully developed mitochondria at anther dehiscence (Hoekstra 1979; Hoekstra 1983). It may be possible to utilise fully developed mitochondria to conduct protein synthesis which is essential for germination and tube growth (Hoekstra and Bruinsma 1979). Both functions were attributed with promotion of germination within the anther prior to dehiscence. However, this had been occurred pre-anthesis at just 1˚C below critical temperature. It may be an indication of the critical temperature. The three types of damage were observed in the sensitive genotype ICC 5912 five days after exposure to high temperature stress.

The number of pollen grains per flower in the heat tolerant and heat sensitive genotypes was reduced with increasing temperature, but pollen grains of the sensitive genotype ICC 5912 only produced sterile pollen at 35/20˚C. The variation in pollen production per flower observed under optimum temperature is likely due to genotype differences (Palmer *et al*. 1978). High temperature (35/20˚C) clearly reduced pollen production and pollen fertility. To confirm this statement, pollen number and % pollen viability were re-plotted in Fig. 2. There was a strong positive relationship (R^2 = 0.85) between pollen production and % of pollen fertility which showed that reduced pollen production was associated with low % pollen fertility regardless of genotype. A decline in pollen fertility with reduced pollen production per flower was also confirmed in groundnut high temperature (De Beer 1963).

The sensitive genotype showed normal and abnormal pollen tubes at 34/19 ˚C during *in vitro* germination. Indehiscent anthers were observed in ICC 5912 during pollen collection at 34/19˚C (supplement Fig. 1c). At anthesis, the relative humidity around the anthers might be reduced and the locules may lose more water by evaporation (Keijzer 1983). This can result in indehiscence due to the wall thickening mechanism of the endothecium (Keijzer 1983). ICCV 92944 showed release of protoplasm by bursting at 38/23 ˚C. We assume that this negative result happened due to low pollen population and medium. Similar results were experienced in many plant species (Brewbaker and Kwack 1963). Mature pollen grains in Fabaceae generally show higher osmotic potential when desiccated (Baker and Baker, 1979; Shivanna, 2003). Due to higher osmotic potential, the high temperature (38/23˚C) stressed pollen burst with 20% sucrose medium (supplement Fig. 1g). Higher sucrose concentration (> 20%) in the media may help to prevent pollen bursting.

High temperature clearly affects flower production and pod set. The reduction in flower production at high temperature is likely due to slower rates of flower bud initiation, flower bud development and flower bud abortion. ICCV 92944 had fewer flowers with lower pod set (50%) at 38/23˚C. No pod set at 35/20˚C was observed in ICC 5912. These findings confirm that pollen production per flower, pollen viability, pollen germination and pod set was reduced by high temperature. The lack of fertile pollen and lack of pod set at 35/20˚C in ICC 5912 indicates this temperature as the threshold for infertility. In general, the number of pods/plant, filled pods/plant and seeds/plant and plant biomass/plant were reduced under high temperature stress. Wang *et al*. (2006) also noted that chickpea plant biomass and number of seeds/ plant were reduced at 35/16˚C compared with 28/16˚C. Flower number and pod number were also reduced in groundnut at high temperature (> 36˚C) (Prasad *et al*. 2000). In the current study, pod set was associated with heat stress effects on anther development, pollen development and pollen release by anther dehiscence. Studies on cowpea (Ahmed *et al*. 1992), common bean (Gross and Kigel, 1994) and groundnut (Prasad *et al*. 2001) also found that high temperature reduces pod set.

The failure of pod set was apparently related to male sterility rather than stigma receptivity. Stressed pollen (35/20˚C) from ICC 5912 did not germinate on either stressed or non-stressed stigmas of the same genotype or ICCV 92944. Non-stressed pollen of ICC 5912 germinated on the stressed stigmas of ICC 5912, indicating that while the pollen was sterile at 35/20˚C, the stigma of ICC 5912 was receptive. Peet *et al*. (1998) reported that tomato (*Lycopersicon esculentum* Mil.) pollen was sterile while stigma was receptive during reciprocal crossing (29˚C x 25˚C; 25˚C x 29˚C). However, the response of ICCV 92944 was different. Stressed pollen germinated on stressed and unstressed stigmas on both ICCV 92944 and ICC 5912, showing that pollen was fertile and stigmas receptive at 35/20˚C.

Overall, the pollen tubes in the non-stressed stigma were smooth but pollen tubes in the stressed stigma grew with callose deposition, although they continued to grow down to the end of the style at 30 min after pollination. High temperature produced a series of plugs resembling a ladder, along the pollen tube in ICCV 92944 (Fig. 1m). Turgor pressure in the pollen tube may be maintained by the formation of a series of callose plugs[2]. Pressure is needed for tube penetration into the pistil, which provides the pathway for the tubes because new plugs would restore pressure (Dumas and Knox 1983). These plugs function to prevent the backflow of cytoplasm and nuclei in the long pollen tubes (Iwanami *et al*. 1988), but physical stress is known to induce callose formation (Aist 1976).

We conclude that pollen is more sensitive to high temperature than the stigma in chickpea. Consequently, there is a potential for developing screening techniques for heat tolerance in chickpea breeding programs using differences in pollen viability. There is also a possibility of using a pollen selection method in breeding for heat tolerance in chickpea. Its success will depend on genotypic variation in high temperature sensitivity of pollen.

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[2] Callose plug, which form in the back of extending pollen tube, thus limit and contain the path of the cytoplasmic stream inside the tube (Stanley and Linskens, 1974).

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Table 1. Details of temperature regime, pre-anthesis, and post-anthesis of chickpea flower collection under controlled environments

Days	Temp regime (day and night - ºC)	Flower buds collected one day before anthesis to check critical temperature (pollen viability)	Pollen germination and tube growth using medium	Post-anthesis (pod set) observation	Hand pollination to study pollen germination and pollen tube growth
Day 1	29/16	-	-	-	-
Day 2	30/16	-	-	-	-
Day 3	31/16	-	-	-	-
Day 4	32/17	-	-	□	-
Day 5	33/18	□	□	□	-
Day 6	34/19	□	□	□	-
Day 7	35/20	□	□	□	□
Day 8	36/21	□	□	□	-
Day 9	37/22	□	□	□	-
Day 10	38/23	□	□	□	-
Day 11	39/24	□	-	-	-
Day 12	40/25	□	-	-	-

Note: The symbol □ indicates the day of sample collection

Table 2. Effect of high temperature on pollen viability (%) within the anther (using 2% Acetocarmine stain) for chickpea genotype (***, * significant at *P*<0.001, *P*<0.05 and NS-Not Significant)

Geno-types	Temp-erature (day /night) ˚C	Anther-1		Anther-2		Anther-3		Anther-4		Anther-5		Anther-6		Anther-7		Anther-8		Anther-9		Anther-10	
		F	S	F	S	F	S	F	S	F	S	F	S	F	S	F	S	F	S	F	S
ICCV 92944	35/20	86	14	93	7	91	9	91	9	93	8	94	6	93	7	90	11	95	9	91	9
ICCV 92944	40/25	30	70	26	74	15	85	13	87	5	95	12	88	0	100	5	95	10	90	0	100
ICC 5912	35/20	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
ICC 5912	40/25	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
Genotype effect																					
ICCV 92944		58	42	59	41	53	47	52	48	49	51	53	47	47	54	48	53	52	48	46	55
ICC 5912		0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100	0	100
Temperature effect																					
35/20		43	57	46	54	46	54	46	55	46	54	47	58	47	54	45	55	48	53	46	55
40/25		15	85	13	87	8	93	7	94	3	98	6	94	0	100	3	98	5	95	0	100
LSD(*P*=0.05)																					
Genotype		28	10	19	19	18	7	13	12	9	9	9	9	3	3	9	9	15	15	11	11

	*	*	*	*	*	*	***	***	***	***	***	***	***	***	***	***	*	*	***	***
Temperature	NS	NS	19	19	18	7	13	12	8	9	9	9	3	3	9	9	15	15	11	11
			*	*	*	*	*	***	***	***	***	***	***	***	***	***	***	***	***	***
Genotype x	NS	NS	27	27	25	9	18	18	12	12	13	13	4	4	13	12	21	21	16	16
Temperature			*	*	*	*	*	***	***	***	***	***	***	***	***	***	*	*	***	***

Note: F-Fertile; S-Sterile

Table 3. Evaluation of high and optimum temperature on pollen germination count (total pollen production per flower) for chickpea genotypes (* significant at $P<0.05$ and NS-Not Significant)

Genotype	Germinated pollen		Sterile pollen		Mean of genotype for germinated pollen	Mean of genotype for sterile pollen
	35/20˚C	27/16˚C	35/20˚C	27/16˚C		
ICCV 92944	2569	4850	124	582	3709	353
ICC 5912	0	3450	2254	411	1725	1332
Mean of temperature	1284	4150	1189	496		
LSD (*P*=0.05)						
Temperature	1300 *		542 *			
Genotype	1300 *		542 *			
Temperature x Genotype	NS		767 *			

Table 4. Pollen germination (%) of chickpea at different high temperature regimes (*** significant at $P<0.001$ and NS-Not Significant)

Temperature Regimes (˚C)	Pollen Germinated (PG)		Pollen Non-germinated (PNG)		Mean	
	ICCV 92944	ICC 5912	ICCV 92944	ICC 5912	PG	PNG
27/16	89	85	11	15	87	13
33/18	64	65	36	35	65	35
34/19	60	39	40	61	50	50
35/20	41	0	59	100	20	80
36/21	26	0	74	100	13	87
37/22	22	0	78	100	11	89
38/23	19	0	81	100	9	91
Mean	46	27	54	73		
LSD($P<0.05$)						
Temperature	12 ***		4 ***			
Genotype	6 ***		2 ***			
Temperature x Genotype	17 *		17 *			

Table 5. Floral morphology (%) of chickpea at different high temperature regimes (*** significant at *P*<0.001; * significant at *P*<0.05and NS-Not Significant)

Temperature Regimes (˚C)	No of flowers (F)		% of dry flowers (DF)		% of pod set (PS)		% of seed set		Mean of temperature regimes		
	ICCV92944	ICC5912	ICCV92944	ICC5912	ICCV92944	ICC5912	ICCV92944	ICC5912	DF	PS	F
27	11	10	4	4	96	96	77	87	4	96	10
32	14	4	18	29	82	71	60	55	24	76	9
33	11	4	10	58	91	42	75	42	34	66	7
34	6	5	28	60	72	40	45	50	44	56	5
35	4	5	29	100	71	0	71	0	65	35	4
36	6	4	82	100	18	0	18	0	90	9	5
37	5	3	80	100	20	0	20	0	90	10	4
38	2	4	50	100	50	0	25	0	75	25	3
Mean of genotype	7	5	38	69	62	31	49	29			
LSD(*P*<0.05)											

Temperature	2***	9***	9***	21***
Genotype	1***	4***	4***	10*
Temp x Genotype	NS	12***	12***	NS

Table 6. Pod characters (per plant) and biomass (per plant) of chickpea at high temperature stress (*** significant at $P<0.001$; * significant at $P<0.05$and NS-Not Significant)

Genotypes	Treatment	Total no of pods	No filled pods	No of seeds	Biomass (g/plant)
ICCV 92944	Control	38	33	35	13.1
ICCV 92944	Heat stress	15	14	15	6.3
ICC 5912	Control	44	40	39	17.4
ICC 5912	Heat stress	14	8	11	15.6
Mean Control		41	37	37	15.3
Mean Heat stress		15	11	13	10.9
LSD (*P*=0.05)					
Temperature		9***	8***	8***	3*
Genotype		NS	NS	NS	3***

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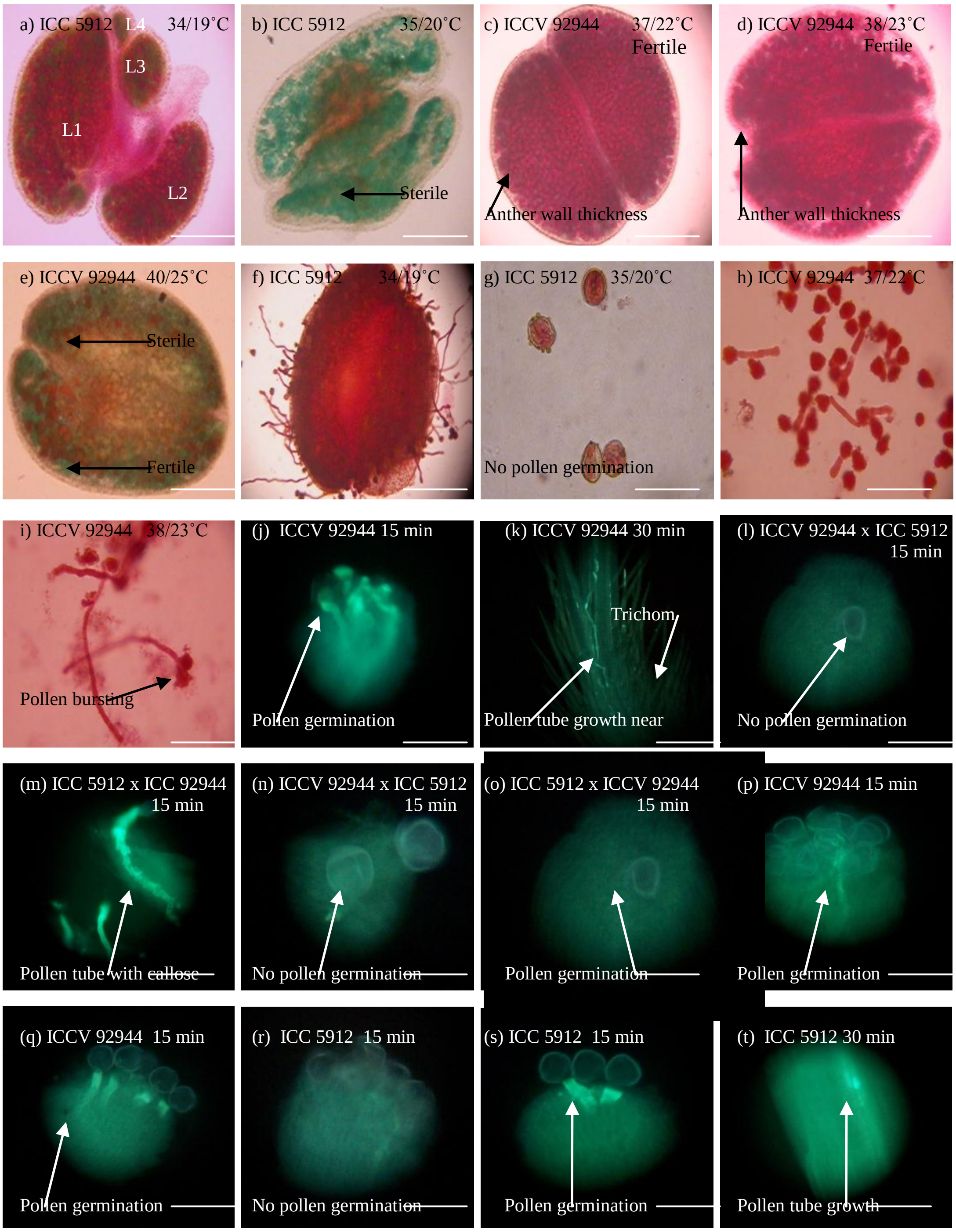
Fig. 2. Relationship between pollen production per flower and % pollen germination.

Supplementary Fig. 1. (a) to (e) Anther-Pollen fertility using Alexander's stain (Fertile pollen grains are red; Sterile pollen grains are green) (L – Locules) (f) to (i) *In vitro* pollen germination after 60 min incubation (Bars = 10 µm)



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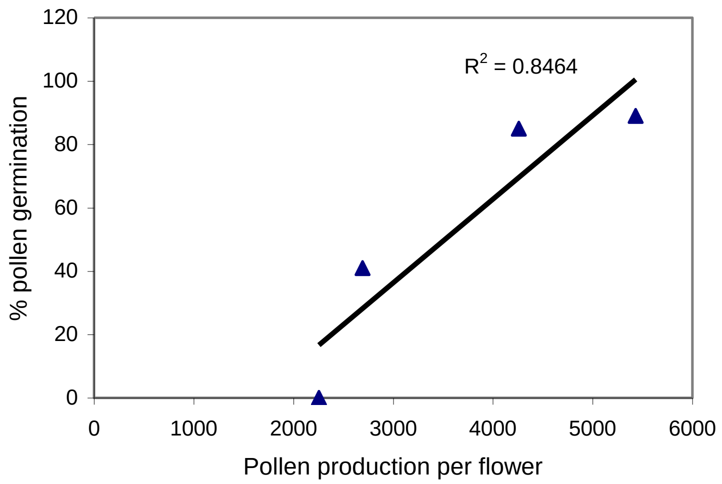
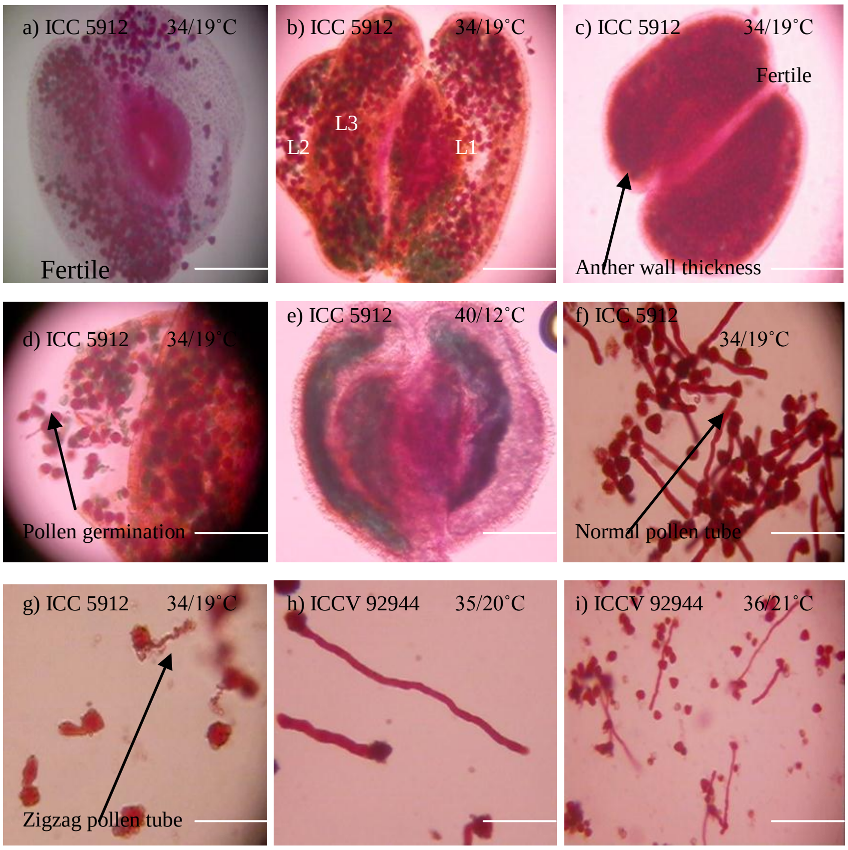


Fig. 2. Relationship between pollen production per flower and % pollen germination (pooled data of 27/16˚C and 35/20˚C day/night of ICCV 92944 and ICC 5912 from Table 3 and Table 4)



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