Reproductive biology of chickpea response to heat stress in the field is associated with the performance in controlled environments

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Running Title: Reproductive biology of chickpea response to heat stress

Abstract

High temperature during reproduction is a major factor limiting the yield of chickpea (*Cicer arietinum* L.). Observations in the field from late season experiments (Feb-

May) and various high temperature regimes generated in controlled environments showed clear genetic variation in male reproductive tissue (anther and pollen), its function (pollen germination and tube growth) and pod set. Greater pod setting ability of heat tolerant genotypes (ICC 1205 and ICC 15614) compared to heat sensitive genotypes (ICC 4567 and ICC 10685) was observed in both the field and controlled conditions. Both anthers and pollen showed more structural abnormalities under stress such as changes in anther locule number, anther epidermis wall thickening and pollen sterility, rather than function (e.g. *in vivo* pollen tube growth). The critical temperature for pod set was $\geq 37°C$ in heat tolerant genotypes (ICC 1205 and ICC 15614) and $\geq 33°C$ for heat sensitive genotypes (ICC 4567 and ICC 10685). Overall, pod set showed greater sensitivity in the controlled environments where a 67% reduction was observed at $\geq 34/19°C$ compared to the control (27/16°C). In the field, a pod set reduction of more than 50% occurred at high ambient day temperature (36°C) and the stigma was still receptive at 40.2/25.5°C. In contrast, under controlled conditions the stigma was still receptive at 35/20°C in four genotypes. Clearly, chickpea pollen grains are more sensitive to high temperature than the stigma in both the field and controlled environments. Among the four genotypes tested, ICC 1205 was the most heat tolerant and ICC 4567 was the most heat sensitive.

Keywords

Anther, heat tolerance, pollen viability, pollen germination, pollen tube growth, pod set

1. Introduction

Generally, high temperatures cause partial to complete male reproductive tissue sterility and significant losses of grain yield in crops. High temperatures during the flowering period influence chickpea grain yield by reducing pod formation and seed set (Wang et al., 2006; Basu et al., 2009). To improve heat tolerance in chickpea,

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a better understanding of how high temperature affects flower production, male structure (anther and pollen), pollen function (pollen germination and pollen tube growth) and pod set is required.

The optimum temperature range for chickpea crop growth, flowering and pod development is 20 to 28°C (Sivaprasad and Sundrasarma, 1987; Summerfield and Wien, 1980). However, temperature ≥ 30°C causes detrimental effects on seed yield (Summerfield and Wien, 1980). Genetic variation in pod set and seed yield per plant at high temperatures was studied in a controlled environment. Wang et al., (2006) showed that high pre-anthesis temperature produced lower pod set compared to post-anthesis heat stress. Devasirvatham et al., (2010) found that pollen sterility is one of the reasons for lower pod set during pre-anthesis high temperature stress. Recently heat tolerant genotypes were identified from field screening in India (Krishnamurthy et al., 2011; Upadhyaya et al., 2011). Morphological, quantitative and qualitative traits were studied in the field screening. However the role of male tissue, its function and grain yield is unclear.

Poor pod set and grain yield in chickpea can result from the high temperature stress during pre- and/or post-anthesis development. Low pollen viability, indehiscent anthers and other anther abnormalities are related to poor pod set following high temperature during pre-anthesis period (Sakata and Higashitani, 2008). The effects of high temperature stress during post-anthesis are also associated with poor pollen germination, tube growth and fertilisation (Gross and Kigel, 1994; Prasad et al., 2001; Kakani et al., 2002). Earlier work has shown that pollen viability and pod set in chickpea is affected at 35/20°C under controlled environments (Devasirvatham et al., 2010). Under controlled conditions, pollen production is reduced at 35/20°C thus demonstrating that pollen is more sensitive to high temperature in chickpea

(Devasirvatham et al., 2012). *In vitro* pollen germination and tube growth studies of chickpea showed that 35˚C and 45˚C reduced germination as compared to that observed at 25˚C (Jaiwal and Mehta, 1983). Kumar et al. (2012) found that oxidative stress expressed as lipid peroxidation and hydrogen peroxide content in the leaves of heat sensitive chickpea genotypes creates failure of fertilization in controlled environments.

Under semi-arid field conditions in south India, chickpea anthers dehisce between 08:00 and 10:00 h, followed by pollination and fertilization. Therefore, high temperature during anthers dehiscence, pollen release and germination may severely affect male reproductive tissue more than ovules (Peet and Willits, 1998). High temperature during flowering also reduced fertility in male rather than female structures in other legumes (cowpea, bean and groundnut) (Hall, 2004). However, the relationship between chickpea response to high temperature in the field and controlled environments is currently unknown. The aim of this research was to determine chickpea response to high temperature by studying pollen fertility through pollen staining, *in vitro* pollen germination, *in vivo* pollen germination and tube growth and pod set under high ambient temperature stress during the reproductive stage in the field and in the controlled environments and to detect genotypic differences.

2. Materials and methods

2.1. Field experiments

Two field experiments were conducted with four chickpea genotypes, two heat tolerant (ICC 1205 and ICC 15614) and two heat sensitive (ICC 4567 and ICC 10685) at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), (17.53^{o}N; 78.27^{o}E; 545 m), Patancheru, India in 2011. The genotypes were selected based on the grain yield and heat tolerance index (HTI) from the

reference collection of 280 genotypes for heat tolerance screening by Krishnamurthy et al. (2011). A randomised complete block design with four replications was used in the late season planting (Feb) on a Vertisol soil to utilise the high temperature stress at the reproductive stage. Seeds of the four genotypes were sown with a plant spacing of 60 × 15 cm. Seeds were treated with fungicide, 0.5% Benlate® (E.I. DuPont India Ltd., Gurgaon, India) + Thiram® (Sudhama Chemicals Pvt. Ltd., Gujarat, India) mixture. Two seeds per hill were sown and these were later thinned to one seedling. A 4 m long row was considered as a replication plot. The phenology of the four genotypes was different. To overcome this issue, two staggered sowings (14 days apart) were planted. The crop received irrigation on 0, 20, 28, 35, 45, 55 and 65 days after sowing. The experiments were maintained weed free by manual weeding. Insecticide was sprayed for pod borer (*Helicoverpa armigera*) when the critical threshold was reached.

The samples for pollen fertility and *in vivo* pollen germination were collected between 50% of flowering stage and 10 consecutive days of maximum reproductive period. Pod set was subsequently recorded. The maximum and minimum temperature of the growing period was recorded daily. The vegetative period was exposed to respective maximum and minimum temperatures of 30-35˚C and 17-20˚C. The maximum and minimum temperature varied between 35-40.4˚C and 17-27˚C during the reproductive period, respectively. The procedures of sample collection, pollen staining to identify fertile pollen grains, *in vitro* pollen germination and *in vivo* pollen germination used to assess the field experiments were similar to those used under controlled conditions in Devasirvatham et al. (2012).

2.2 Controlled environments

Two controlled environment experiments were conducted with four chickpea genotypes (ICC 4567; ICC 10685; ICC 1205 and ICC 15614) at ICRISAT, Patancheru, India in 2010 and 2011 with five replications. Soil preparation, sowing, maintenance of the controlled environment growth room, and the movement of plants from the control to the high temperature growth room including high temperature treatments was explained in Devasirvatham et al. (2012).

2.3 Pollen fertility studies using Alexander and Acetocarmine stains

2.3.1 Field

The fertility of pollen at high temperature was confirmed using staining. The effect of high temperature (38/25.2˚C) was studied in flower buds (1 DBA) (pre-anthesis) and open flowers (anthesis) with Alexander's stain to determine pollen fertility. Five flower buds and five open flowers were collected between 08:00 and 08:15 h to examine pollen fertility. Anthers were stained with Alexander's stain (Alexander, 1969) and examined under a compound microscope as shown in Fig 1. The fertile pollen grains inside the anthers were stained red whilst the sterile pollen grains remained green. To confirm the fertility, the anthers were squashed from 1 DBA flower buds and stained with 2% acetocarmine. Pollen grains from open flower samples were stained with 2% acetocarmine (data not shown).

2.3.2. Controlled environments

The effects of one day exposure to day/ night temperatures of 31/16˚C and 40/25˚C one day prior to anthesis were studied to determine the critical temperature. Five flower buds were collected between 08:00 and 08:15 h to examine pre-anthesis pollen fertility. Anthers were stained with Alexander's stain and examined under a compound microscope. The results are discussed in Table 3.

2.4 *In vitro* pollen germination and tube growth

2.4.1 Field

The flower samples were collected at 35.2/24˚C; 36.4/23.4˚C; 37.1/21.8˚C and 38/25.2˚C day/ night temperatures. Open flowers were collected in the morning between 08:00 and 08:15 h to examine *in vitro* pollen germination and pollen tube growth. The *in vitro* pollen germination was terminated after 60 min incubation by adding a drop of 2% acetocarmine stain. The procedure of pollen germination assessment was explained in Devasirvatham et al. (2012). Two replications per temperature treatment were used to calculate pollen germination.

2.4.2. Controlled environments

The percentage of germinated pollen grains was calculated to estimate the effect of temperature regime (33/18˚C to 40/25˚C) on pollen viability. The flowers were collected daily between 08:00 and 08:15 h from the 33/18˚C and 40/25˚C treatments to examine *in vitro* pollen germination and pollen tube growth. The results are discussed in Table 3.

2.5. *In vivo* pollen germination and tube growth

2.5.1. Field

Hand pollinations were carried out to obtain *in vivo* pollen germination and tube growth. Crosses (stressed stigma x stressed pollen) were conducted at 38/25.2˚C and 39/25.2˚C day/ night temperature treatments. Reciprocal crosses (stressed stigma x non-stressed pollen 40.2/25.5˚C x 27/16˚C) were carried out to determine the stigma receptivity for high temperature stress. In summary, the following crosses were made

  i. Stressed stigma x stressed pollen within the same genotype

 ii. Stressed stigma x non-stressed pollen within the same genotype

Seven flowers were collected 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.

2.5.2. Controlled environments

Hand pollination was carried out at 35/20˚C day/night temperature to obtain *in vivo* pollen germination and tube growth. Reciprocal crosses (stressed x non-stressed; non-stressed x stressed) were carried out to determine which tissue; pollen or stigma is most responsive to high temperature stress. The following crosses were made

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

    iv. Non-stressed stigma (27/16˚C) x stressed pollen (35/20˚C) within the same genotype

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

A similar procedure of *in vivo* pollen germination to that used in the field was followed in the controlled environments.

2.6. Pod set

2.6.1. Field

Pod set was observed at 50% of flowering, and for 10 consecutive days post this date by tagging 20 flowers daily. Pod set was counted for each flower seven days after anthesis.

2.6.2. Controlled environments

Flowers grown between 32/17˚C and 40/25˚C were tagged to observe the pod set.

2.7. Ovule observation

The numbers of ovules in an ovary were counted in the field at 39.4/27.2˚C and compared with the non-stressed treatment (27/16˚C) of the controlled environment experiment.

2.8. Statistical analysis

Analysis of variance (ANOVA) was performed for flower and pod data, pollen germination percentage of different temperature regimes using Genstat 12th Ed. VSN International Ltd. For both experiments, two way ANOVA (genotype × temperature) was conducted with two replications for pollen germination (%). Similarly two way ANOVA (genotype × temperature) was performed for floral morphology (%) with five replications to study the difference between temperature treatments in the controlled environments. For grain yield, pod characters and plant biomass at harvest in the field was studied with four replications using one way ANOVA. A curve relating % pod set to high temperatures (day/night) was generated.

3. Results

3.1. Field response

3.1.1. Pollen fertility studies using Alexander and Acetocarmine stains

The genotype ICC 4567 exhibited abnormal anthers when exposed to 38/25.2˚C before anthesis (Fig 1a) and during anthesis (Fig 1e, f). Increased numbers of locules were observed before anthesis (Fig 1a) and at anthesis (Fig 1e). Fig 1a and Figs 1e, f showed the epidermis wall thickening in response to increasing temperature. The epidermis wall thickening was a likely cause of indehiscence and partial dehiscence at anthesis (Fig1a). A mixture of fertile and sterile pollen grains was observed after exposure to high temperature during pre-anthesis (Fig 1a) and this coincides with appearance of pronounced anther indehiscence at anthesis (Fig 1e). However, dehiscent anthers mixed with fertile

and sterile pollen grains were observed in ICC 4567 during anthesis at 38/25.2˚C. ICC 10685 produced fertile pollen grains (Fig 1b) but epidermis wall thickening and reduced pollen number was observed after high (38/25.2˚C) pre-anthesis temperature. On the other hand, the line ICC 10685 produced dehiscent anthers at anthesis (Fig 1g). Fertile pollen grains were found in the anthers of ICC 1205 (Fig 1c) and these dehisced at 38/25.2˚C during anthesis (Fig 1h). Although the anthers of ICC 15614 dehisced at 38/25.2˚C during anthesis (Fig 1i), the anther had few sterile pollen grains. With acetocarmine stain all genotypes showed similar results to those observed using Alexander's stain (data not shown). In addition, the pollen cytoplasm of ICC 10685 appeared to be released at 38/25.2˚C during anthesis (data not shown). Therefore, both stains confirmed the fertility of pollen under high temperature.

3.1.2. *In vitro* pollen germination and tube growth

In vitro pollen germination and tube growth was observed at 35.2/24˚C; 36.4/23.4˚C; 37.1/21.8˚C and 38/25.2˚C day/ night temperatures. Increasing temperature gradually reduced pollen germination (%) (Table 1). Pollen germination in ICC 4567 was 7%, 6% and 0% at 35.2/24˚C, 36.4/23.4˚C and 37.1/21.8˚C, respectively (Fig 2). There were also changes in pollen grain size observed and the stain entered into the structurally abnormal (size variation) pollen grain. In ICC 10685 germination decreased from 16% (35.2/24˚C) to 11% (38/25.2˚C). In ICC 1205 and ICC 15614 pollen germination was 55% at 35.2/24˚C, however, this decreased to 33% in ICC 1205 and 22% in ICC 15614 at 38/25.2˚C. Percentage pollen germination across genotypes was lower ($P < 0.001$) with every 1˚C increase ($P < 0.05$), but no difference was measured between genotypes and temperature when samples were examined at 60 min incubation period.

3.1.3. Pod set

Genotypic differences observed for *in vitro* pollen germination under high temperature were consistent in pod set (Figs 3 and 4). The temperature range in staggered sowing-1 was 35.6/17.4 to 37.5/17˚C (day/night). The tolerant genotypes ICC 1205 and ICC 15614 produced 65% and 41% of mean pod set respectively. In contrast, the sensitive genotypes ICC 4567 and ICC 10685 produced 4% and 1%, respectively. The temperature range in staggered sowing-2 was 33.7/23.4 to 39/25.2˚C day/night temperatures. Within this range, ICC 1205 produced 50% pod set, followed by ICC 15614 (39%). Pod set on the sensitive genotypes ICC 4567 and ICC 10685 was 3% and 1%, respectively. In both sowings, ICC 1205 set more pods (65%; 50%) than other genotypes followed by ICC 15614 (41%; 39%). The sensitive genotype ICC 4567 produced a pod set of 4% and 3%, for the first and second sowing, respectively. ICC 10685 was the most heat sensitive genotype which with 1% pod set in both sowings.

3.1.4. *In vivo* pollen germination and tube growth

i. Stressed stigma x stressed pollen within the same genotype (38/25.2 x 38/25.2˚C; 39/25.2 x 39/25.2˚C)

Thirty minutes after hand pollination at 38/25.2˚C in the field the pollen of ICC 4567 did not germinate on the stigma (Fig 5a). At 39/25.2˚C the style of the same genotype was shortened (Fig 5h). The pollen of ICC 10685 germinated at 38/25.2˚C and 39/25.2˚C but had more callose formation in the style tissue nearest the stigma (Fig 5c) and less callose development at the base of the style (Figs 5i). In contrast, pollen germinated and had smooth pollen tube growth at the base of the style in ICC 1205 under both temperature regimes (38/25.2˚C; 39/25.2˚C) (Figs 5d and 5j). Similar responses were observed in ICC 15614 (Figs 5f and 5l).

ii. Stressed stigma x non-stressed pollen within the same genotype (40.2/25.5˚C x 27/16˚C)

In three genotypes (ICC 10685; ICC 1205 and ICC 15614), the non-stressed pollen (27/16˚C) germinated on the stressed stigma at 40.2/25.5˚C and the pollen tube reached the base of the style with little callose development on the style (Figs 5m, 5n and 5o). The stressed stigma was clearly receptive in all three genotypes. There was no sample for ICC 4567.

3.1.5. Ovule observation

The number of ovules were counted at high temperature (39.4/27.2˚C) and compared with the non-stressed treatment (27/16˚C) of the controlled environment experiment. ICC 4567 had five ovules in an ovary (Fig 6b) under high temperature stress whereas this genotype normally produced four ovules under non-stressed conditions (27/16˚C) (Fig 6a). The size of the ovary was therefore larger than other genotypes (Fig 6c). Other genotypes (ICC 10685; ICC 1205 and ICC 15614) produced three ovules in an ovary in the stressed treatment, compared with only two ovules in the non-stressed treatment (Figs 6d, e, f).

3.1.6. Grain yield and biomass at high temperature in the field

High temperature significantly influenced pod number, filled pod number, seed number and grain yield per plant ($P<0.001$). Significant differences among genotypes were observed at high temperature (Table 2). The results showed that ICC 1205 and ICC 15614 were heat tolerant and the genotypes ICC 4567 and ICC 10685 more heat sensitive. However, the genotypes did not show any significant difference in biomass at maturity.

3.2. Controlled environments response

The overall summary of the pre-anthesis pollen fertility, *in vitro*, *in vivo* pollen germination, and pod set is presented in Table 3 and a more complete description of the responses is available in the following section:

3.2.1. Pollen fertility studies using Alexander's stain

In ICC 4567, a mixture of fertile and sterile pollen grains was observed in the anther at 35/20˚C and 36/21˚C and all pollen grains became completely sterile at 37/22˚C. Epidermis wall thickening occurred from 35/20˚C. Then pollen grains of ICC 10685 were fertile at 36/21˚C but the pollen germinated within the anther. At 37/22˚C, the pollen grains were completely sterile. ICC 1205 pollen grains were fertile at 38/23˚C. A mixture of fertile and sterile pollen grains was observed at 39/24˚C and 40/25˚C. ICC 1205 showed epidermis wall thickening at 38/23˚C. In ICC 15614, pollen grains were fertile and the anther dehisced at 39/24˚C. The number of flowers produced by this genotype reduced after 36/21˚C.

3.2.2. *In vitro* pollen germination and tube growth

High temperature reduced pollen germination % which indirectly implied pollen fertility. At 37/22˚C, pollen germination was zero in ICC 4567, ICC 10685 and ICC 15614. ICC 1205 also produced completely sterile pollen at 38/23˚C. Differences were observed in pollen germination % between genotypes ($P < 0.05$) and between temperature regimes ($P < 0.001$). However, the genotype and temperature regimes interaction was non significant.

Genetic variation in pollen tube growth among the genotypes was determined. At 35/20˚C, ICC 4567 had abnormal zigzag pollen tubes. The stain entered into the shrunken and structurally abnormal pollen and the release of protoplasm was observed in this genotype at 35/20˚C. ICC 10685 produced abnormal tubes at 36/21˚C. At 37/22˚C, intact pollen grain (cell walls intact) was found but the contents

of pollen started to stream out through an aperture which resembled a pollen tube. Pseudo-germination, wrinkled pollen grain and tri-apertures germination were typical abnormalities under high temperature stress (38/23˚C). ICC 1205 pollen germinated at 37/22˚C and anthers were indehiscent. The release of protoplasm and tri-apertures germination was found at 38/23˚C. ICC 15614 was observed to have a bulbous pollen tube tip at 36/21˚C compared to a cylindrical tip in the control temperature (27/16˚C). Shrunken and round, fully and partially stained pollen grains were observed at 37/22˚C in ICC 15614. Indehiscent anthers were also found in this genotype at 37/22˚C and tri-aperture germination was observed at 38/23 ˚C.

3.2.3. *In vivo* pollen germination and tube growth

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

At 35/20˚C, stressed pollen germinated on the stressed stigma in four genotypes and the pollen tube grew to the base of the style. This indicated that the stressed pollen from four genotypes were fertile. ICC 15614 produced more callose deposition in the stigma.

iv. Non-stressed stigma (27/16˚C) x stressed pollen (35/20˚C) within the same genotype

In two genotypes (ICC 4567 and ICC 10685) stressed pollen (35/20˚C) did not germinate on the non-stressed stigma (27/16˚C). In ICC 1205 the stressed pollen germinated and produced a smooth pollen tube in the non-stressed stigma. However, the pollen of ICC 15614 germinated and more callose deposition was noted in the style tissue close to the stigma. Hence, the pollen grains from both genotypes (ICC 1205 and ICC 15614) were fertile. The pollen tube in this genotype entered the ovary.

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

The non-stressed pollen germinated in the stressed stigma and produced smooth pollen tube growth in all four genotypes. The high temperature stress reduced the style length in the genotype ICC 4567 at 35/20˚C.

3.2.4. Pod set

Compared to the non-stressed control, the high temperature treatments reduced flower number and % pod set. The number of flowers was reduced from 22 at 32/17˚C to 2 at 38/23˚C ($P < 0.001$). No pods were set at 36/21˚C in ICC 4567 and at 34/19˚C in ICC 10685. In ICC 1205, 5% pod set occurred at 37/22˚C and was zero at 38/23˚C. 13% pod set occurred at 36/21˚C and became zero at 37/22˚C in ICC 15614. Overall, % pod set was reduced from 86% (27/16˚C) to 1% (37/22˚C). Differences ($P < 0.001$) were found in genotypes, temperature regimes and there was an interaction between temperature and genotypes.

Fig 7 was replotted from pod set (data not shown) to identify the critical temperature for % pod set in chickpea under controlled environments. The critical temperature for pod set was 34/19˚C because pod set was reduced by 13% at this temperature compared to 33/18˚C. A 67% of reduction in pod set occurred at 34/19˚C compared to the optimum temperature (27/16˚C). Overall, there was a strong negative relationship ($R^2 = 0.95$) between pod set and high temperature, such that pod set was reduced by 39% per 1˚C above the threshold temperature.

3.3.5. Comparison of field and controlled environment pod set at different temperature

The adverse effect of high temperature on % pod set was compared in both field and controlled environments (Fig 8). The aim was to identify the critical temperature for pod set in chickpea under high temperature. Therefore Fig 8 was replotted from pod set from controlled environment, Figs 3 and 4. The critical

temperature for pod set was ≥38˚C in ICC 1205; ≥37˚C in ICC 15614; ≥34˚C in ICC 4567 and ≥33˚C in ICC 10685. There were clearly differences among genotypes in the critical temperature for pod set with temperatures ranging from 33 to 38˚C.

4. Discussion

The data showed that % pod set in chickpea was reduced at high temperatures (day/night). Factors producing lower pod set were the influence of high temperatures on the flower production, anther dehiscence, pollen viability, pollen germination and pollen tube growth. An increased number of locules, epidermis wall thickening, and a mixture of fertile and sterile pollen grains were observed under high pre-anthesis temperature stress in the heat sensitive genotypes (ICC 4567 and ICC 10685) in the field. At anthesis, sensitive genotypes produced indehiscent anthers, epidermis wall thickening of anthers, released protoplasm from the pollen grain and pollen germination within the anther. Further investigation of indehiscent anthers should consider factors affecting microclimatic conditions within the anther. Warrag and Hall (1984) and Porch and Jehn (2001) reported changes in the locule numbers and epidermis wall thickening in anthers due to failure of stomium opening and anther cell differentiation under high temperature stress in cowpea and bean.

Genetic variation in pollen fertility was observed under high temperature stress. In all genotypes, shrunken and structurally abnormal deformities of pollen occurred in both the field and controlled environment experiments at high temperature. ICC 10685 produced intact pollen grain however, pollen tubes were abnormal (zigzag growth) and pseudo-germination was observed. The pollen grains were wrinkled and tri-aperture germination occurred at high temperature under controlled conditions. The genotype ICC 4567 showed abnormal tubes (zigzag growth) with protoplasm starting to stream out of the pollen grains due to osmosis

(Kroon et al., 1974). At high temperature, i*n vitro* pollen tube abnormalities such as thinner, zigzag tubes with stunted growth was also observed in *Brassica napus* (Young et al., 2004), and in groundnut (Prasad et al., 2001). Reduced *in vitro* pollen germination (44%) at > 35˚C was also reported in groundnut (Kakani et al., 2002); 50% reduction at 38˚C in soybean (Koti et al., 2005) and 52% reduction in bean at 32/27˚C (Porch and Jehn 2001). These data suggest that pollen viability is the main cause of sterility in chickpea under high temperature stress at anthesis.

The critical temperature for pod set in heat tolerant and sensitive genotypes were clearly identified. Generally the pod set was reduced under high temperatures. Devasirvatham et al. (2010) found that ~ 50% of pod set reduction in chickpea at 35/20˚C compared to the optimum temperature (28/16˚C). Wang et al. (2006) calculated that pod production was decreased by 34% for Myles and 22% for Xena chickpea cultivars at 35/16˚C compared with the control (20/16˚C). Therefore, hot days ($\geq$ 34˚C) combined with warm nights ($\geq$ 19˚C) can potentially reduce pod set in chickpea. In general, the threshold temperature for chickpea pod set under high temperature is $\geq$ 34/19˚C. These findings are similar to those of Prasad *et al*. (2001) who showed that the critical pre-anthesis flower bud temperature in groundnut was $\geq$ 33˚C, above which pod set reduced by 6% pod set per 1˚C. Similarly, an increase in the seasonal temperature of 1˚C reduced chickpea yield by 53 – 300 kg/ha in different regions of India (Kalra et al., 2008).

In our study high temperature decreased flower production and increased flower abortion. This is consistent with an earlier report of flower abortion in chickpea at > 30˚C (Sinha, 1977). Although chickpea is an indeterminate grain legume and plant growth continues after flowering, given sufficient moisture flower

number reduced in our study under high temperature. Flower number was also reduced in groundnut under high temperature (Prasad et al., 1999).

In the field, stressed pollen did not germinate on the stressed stigmas of ICC 4567. Though, stressed pollen was observed to germinate on the stressed stigmas of ICC 1205 and ICC 15614 indicating better heat tolerances in these genotypes. The *in vivo* germination indicates the sensitivity of pollen fertility for heat tolerant and sensitive genotypes under high temperature. The non-stressed pollen grains of ICC 10685; ICC 4567 and ICC 15614 germinated and produced smooth pollen tube growth on the stressed stigmas (40.2/25.5°C) of the same genotypes. Clearly the stigmas remain receptive at high temperature in chickpea.

However, short style was observed in heat sensitive genotypes such as ICC 4567 in both field (39°C) and controlled environments (35°C). Plants were continuously stressed for more than 10 days in the field and seven days in the controlled environments during flowering. At 39°C in field this genotype also showed ovule and ovary abnormalities. These results suggested that high temperature initially reduces pollen fertility (≥3 days) and extended exposure to high temperature (≥7 days) eventually effects the stigma and ovule. Female abnormalities were observed in wheat at 30°C over 3 continuous days (Saini et al., 1983). However, deformity of male and female reproductive tissue depends up on the critical temperature of the crop and the level of heat tolerance among genotypes.

In the controlled environment at 35/20°C the stressed pollen grains of ICC 4567 and ICC 10685 did not show consistency in germination on the stressed or non-stressed stigmas due to low pollen fertility (%). However, pollen germination on the stigma of the tolerant genotypes (ICC 1205 and ICC 15614) was much higher and more consistent. Therefore, these genotypes have stable heat tolerance. Similarly, the

stigmas of bean (Gross and Kigel, 1994) and tomato (Peet et al., 1998) remain receptive at high temperature. Overall, the pollen grain of chickpea was more sensitive to high temperature than the stigma in both the field and controlled environments.

5. Conclusions

Generally, high temperature $\geq 35\,°C$ negatively affects pollen fertility, *in vitro* pollen germination, *in vivo* pollen germination and pollen tube growth thus reducing subsequent pod set. The percentage of *in vitro* pollen germination and tube growth exactly reflects % pod set under high temperature. Similarly high temperature during pre-anthesis also showed clear evidence of poor pollen fertility under heat stress. The threshold temperature for pod set was $\geq 34/19\,°C$. We conclude that microsporogenesis and pollination are the most critical events of flower development in chickpea under high temperature. Therefore, pre-anthesis and anthesis are the most sensitive stages to high temperature in chickpea. Among the four genotypes tested, ICC 1205 was the most heat tolerant genotype and ICC 4567 the most heat sensitive. ICC 10685 was also classified as sensitive but was low yielding in the optimal growing conditions due to a genetically inherent problem. In conclusion, the heat tolerant genotypes identified in this study are suitable for inclusion in breeding programs targeting warmer areas. The materials are also potential parents for developing genetic mapping populations to identify QTLs (Quantitative Trait Loci) linked to heat tolerance.

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Table 1. Pollen germination (%) of chickpea under different temperature regimes (day/ night) in the field ((*** significant at P < 0.001; * significant at P < 0.05)

Temperature regimes (˚C)	Relative Humidity (%)	ICC 4567		ICC 10685		ICC 1205		ICC 15614		Mean	
		PG	PNG	PG	PNG	PG	PNG	PG	PNG	PG	PNG
35.2/24	67/27	7	93	16	84	55	45	55	46	33	67
36.4/23.4	85/41	6	95	12	88	50	50	42	59	27	73
37.1/21.8	76/31	0	100	11	89	46	55	26	77	20	80
38/25.2	72/26	0	100	11	89	33	67	22	78	17	83
Genotype		3	97	13	87	46	54	35	65		
LSD(*P*<0.05)											

Genotype	11***
Temperature	11*

Relative humidity (day/ night) was not statistically analysed. PG - Pollen germinated; PNG - Pollen not germinated

Table 2. Grain yield (g/plant), pod characters (per plant) and biomass (g/plant) of chickpea genotypes at high temperature in the field

Genotypes	Total number	podFilled number	podSeed number	GY	Biomass
ICC 4567	10[C]	3[C]	4[C]	0.4[C]	23.0[a]
ICC 10685	2[d]	1[d]	2[d]	0.2[d]	25.2[a]
ICC 1205	69[a]	65[a]	87[a]	11.7[a]	21.9[a]
ICC 15614	66[b]	62[b]	88[b]	10.5[b]	20.4[a]
Mean ± s.e.	37±7.9	33±7.6	45±9.3	5.7±1.2	22.6±5.4
CV%	43.6	46.4	41.4	42.2	47.5

Means within the column followed by different letters are significantly different (P<0.001)

Table 3. Summary of findings on the effect of high temperature on pre-anthesis pollen fertility, *in vitro*, *in vivo* pollen germination and pod set of chickpea under controlled environments

Observations	ICC 4567	ICC 10685	ICC 1205	ICC 15614
Pre-anthesis pollen fertility was found up to	35/20˚C	36/21˚C	38/23˚C	38/23˚C
Abnormalities during pre-anthesis at high temperature	Mixture of fertile and sterile pollen grains	Pollen germination within the anther	Anther wall thickness	Mixture of fertile and sterile pollen grains
In vitro pollen germination was found at	35/20˚C	36/21˚C	38/23˚C	38/23˚C
Abnormalities during *in vitro* pollen germination at high temperature	Zigzag tube growth and leakage of protoplast	Intact pollen tube growth, pseudo germination and wrinkled sterile pollen grain	Indehiscent anthers	Bulbous tip in the pollen tube
In vivo pollen germination at 35/20˚C: stressed stigma x stressed pollen and non-stressed	No pollen germination on the stigma	No pollen germination on the stigma	Pollen germination and tube growth on the style	Pollen germination and tube growth on the style

stigma x stressed pollen confirmed the results				
Stigma receptivity observed through stressed stigma (35/20˚C) x non-stressed pollen (27/16˚C)	Mostly stigma was receptive. However, short style was observed.	Stigma receptive	Stigma receptive	Stigma receptive
Critical temperature for pod set	34/19˚C	34/19˚C	38/22˚C	37/21˚C

List of Figures

Fig 1. Anthers respond to 38/25.2°C in the field (Sample were stained with Alexander's stain). Figs (a) to (d) are high pre-anthesis temperature and (e) to (i) are high anthesis temperature (open flowers) (Fertile pollen grains are red; Sterile pollen grains are green). (L-Locules) (Bars = 10 μm)

Fig 2. Field response: *In vitro* pollen germination (anthesis) at 37.1/21.8°C a) ICC 4567 – no pollen germination (changes in pollen grain size) b) ICC 10685 – few pollen grains germination c) ICC 1205 – mixture of germinated and non-germinated d) ICC 15614 –germinated pollen grain (Bars = 10 μm)
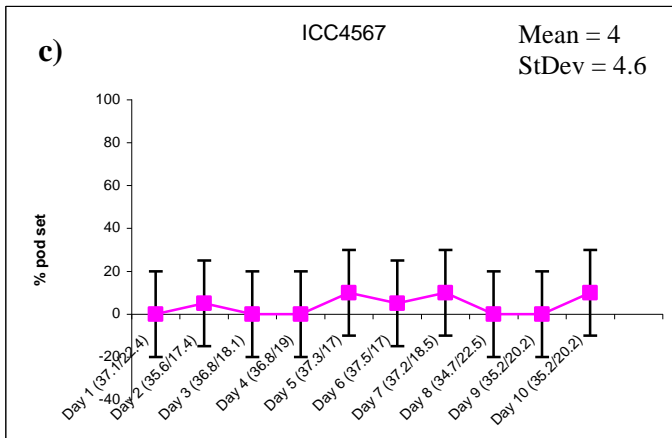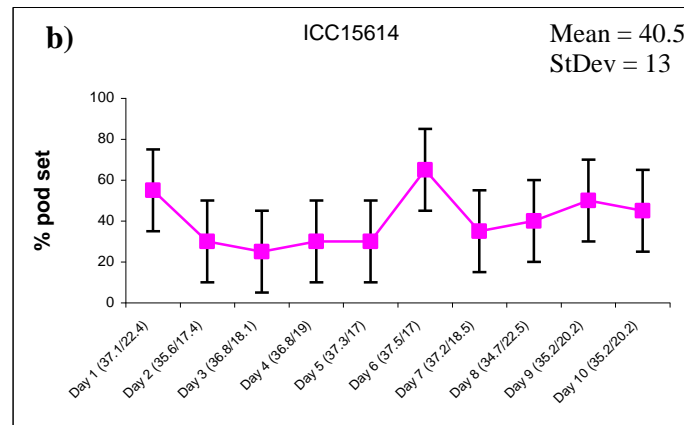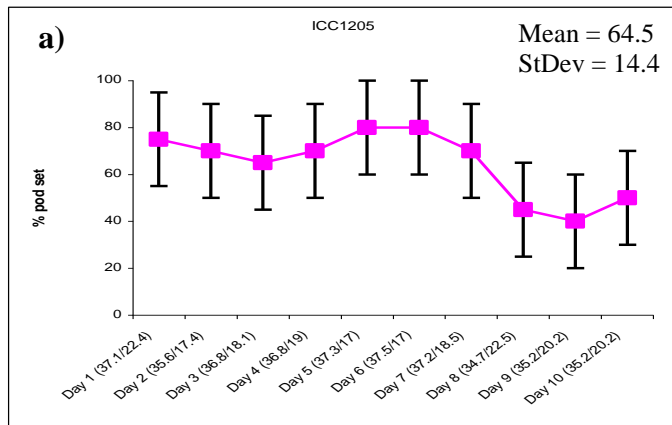
Fig 3. Daily pod set during maximum active period (10 days) of reproductive stage (observation were recorded from 50% flowering date) in the field – Staggered sowing -1 (date of sowing – 4/2/2011; date of harvest – 20/5/2011) Values are mean ± SE (n = 10)

Fig 4. Daily pod set during maximum active period (10 days) of reproductive stage (observation commenced from 50% flowering date) in the field – Staggered sowing -2 (date of sowing – 18/2/2011; date of harvest – 30/5/2011) Values are mean ± SE (n = 10)
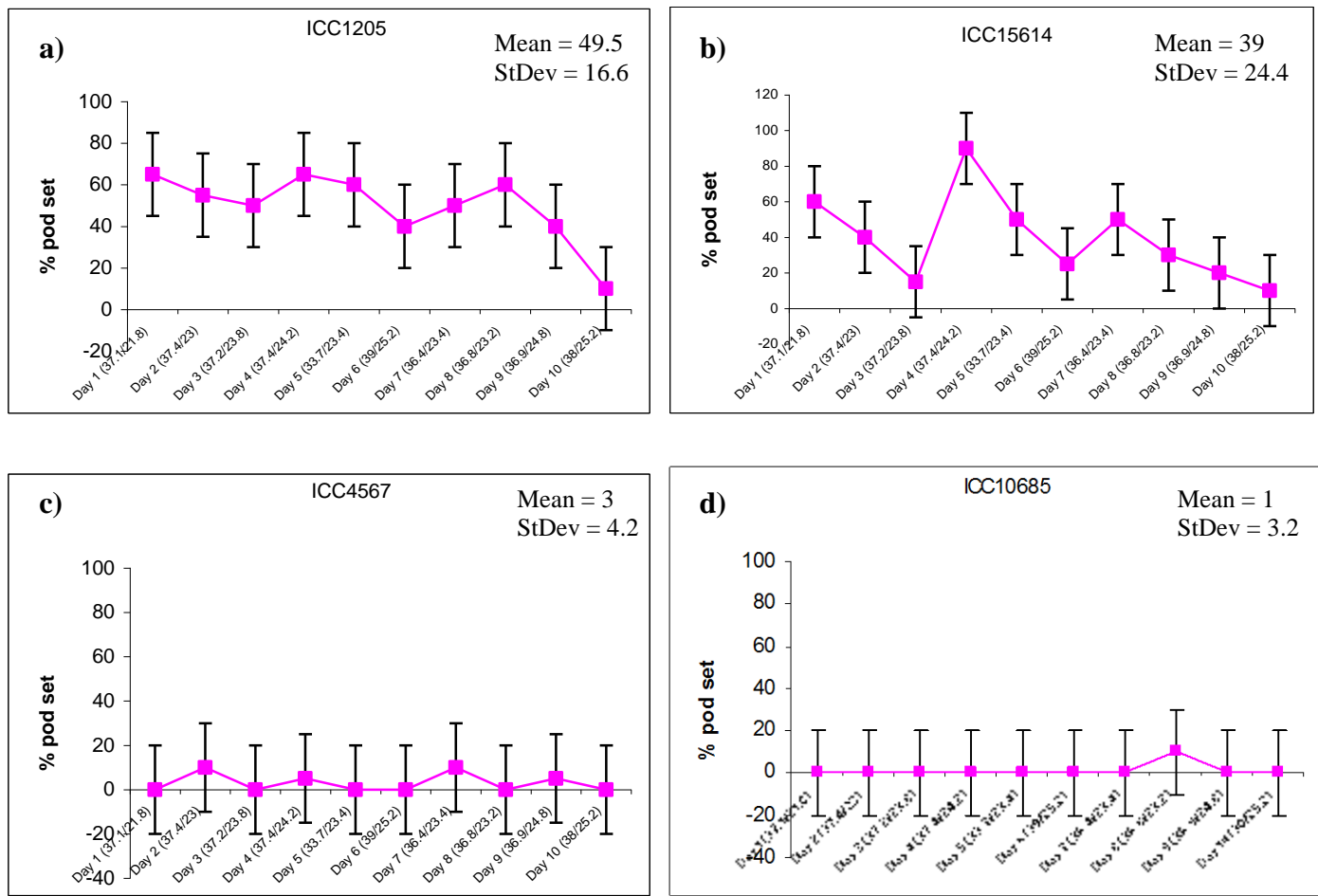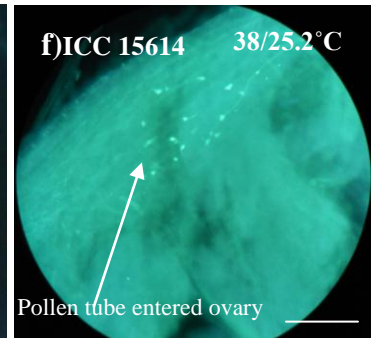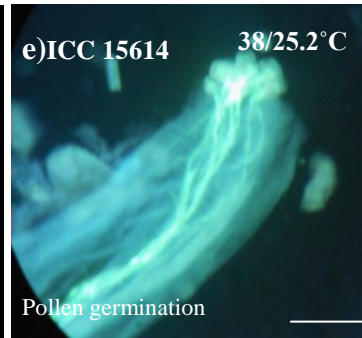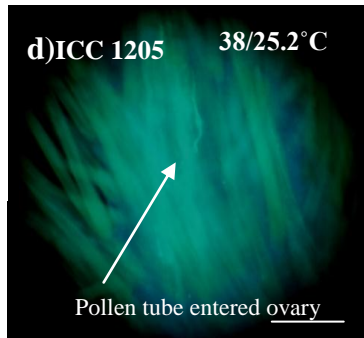
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Fig 6. Numbers of ovules under heat stress (39.4/27.2°C) in the field compared with optimum temperature (27/16°C) under the controlled environments. a) ICC 4567 – 4 ovules at 27/16°C b) ICC 4567 – 5 ovules at 39.4/27.2°C c) ICC 4567 – larger size (4.3mm length) ovary at 39.4/27.2°C (Ovule size was not measured) d) ICC 10685 – 2 ovules at 27/16°C e) ICC 10685 – 3 ovules at 39.4/27.2°C f) ICC 1205 – 3 ovules at 39.4/27.2°C (Bars = 10 μm)

Fig 7. Relationship between temperature and pod set in the controlled environments (Table 4 mean values % pod set were replotted to predict the reduction by every temperature increase by 1˚C using logarithmic regression)

Fig 8. Comparison of the pod set in the field (F) and the controlled environments (GH) under high day temperature

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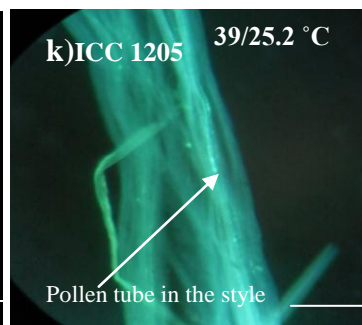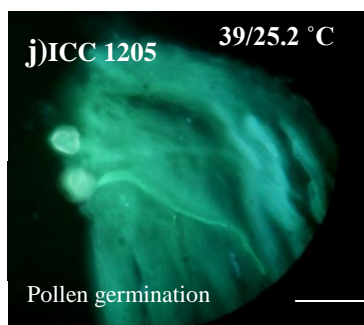
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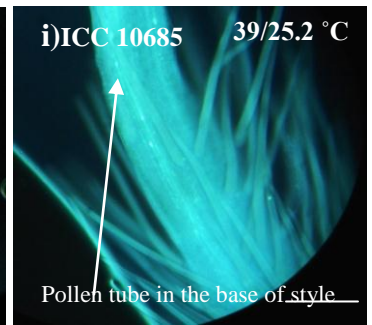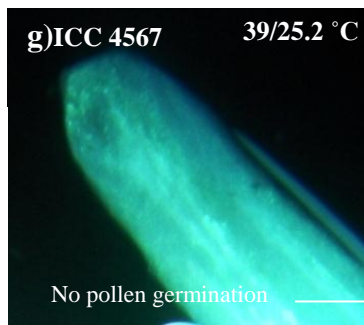
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Stressed stigma x stressed pollen
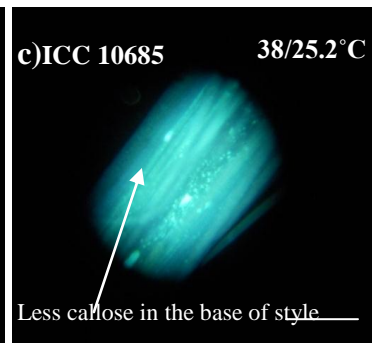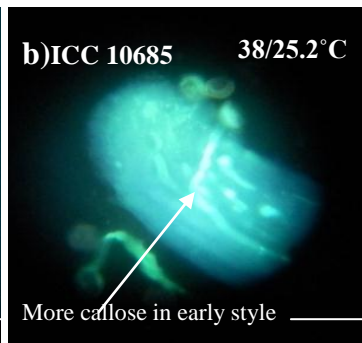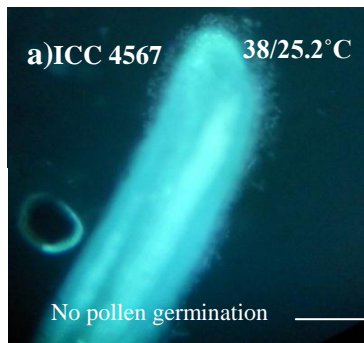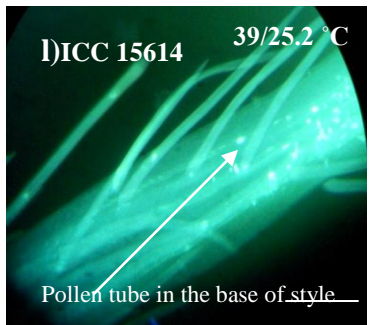
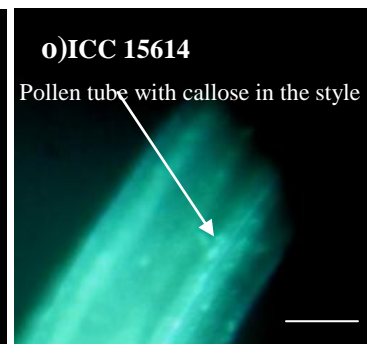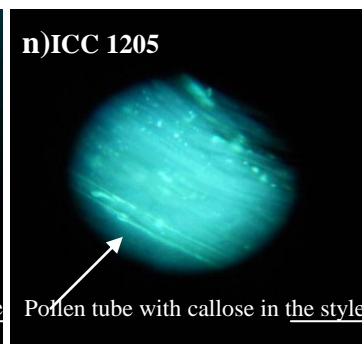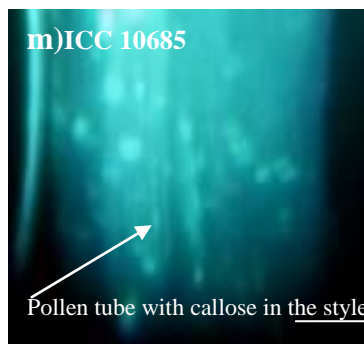Stressed stigma x stressed pollen

Stressed stigma x non-stressed pollen

a)ICC 4567 38/25.2˚C
No pollen germination

b)ICC 10685 38/25.2˚C
More callose in early style

c)ICC 10685 38/25.2˚C
Less callose in the base of style

d)ICC 1205 38/25.2˚C
Pollen tube entered ovary

e)ICC 15614 38/25.2˚C
Pollen germination

f)ICC 15614 38/25.2˚C
Pollen tube entered ovary

g)ICC 4567 39/25.2 ˚C
No pollen germination

h)ICC 4567
Short style

i)ICC 10685 39/25.2 ˚C
Pollen tube in the base of style

j)ICC 1205 39/25.2 ˚C
Pollen germination

k)ICC 1205 39/25.2 ˚C
Pollen tube in the style

l)ICC 15614 39/25.2 ˚C
Pollen tube in the base of style

m)ICC 10685
Pollen tube with callose in the style

n)ICC 1205
Pollen tube with callose in the style

o)ICC 15614
Pollen tube with callose in the style

Fig 5. *In vivo* pollen germination and tube growth in the field under heat stress (Bars = 10 µm)
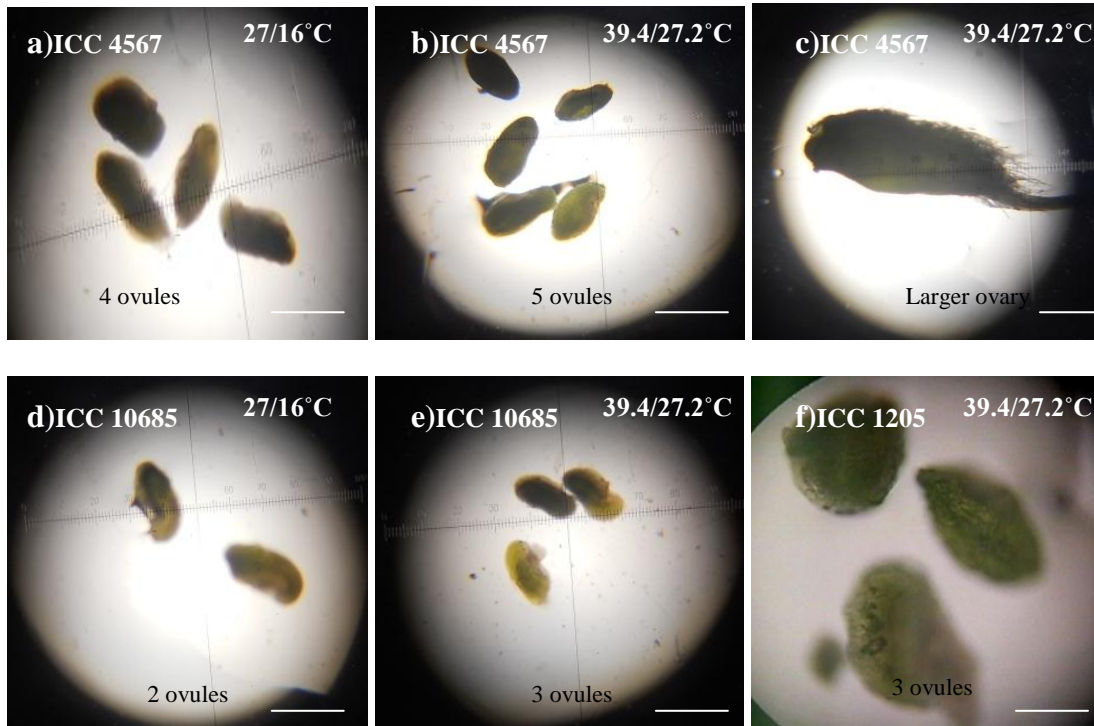

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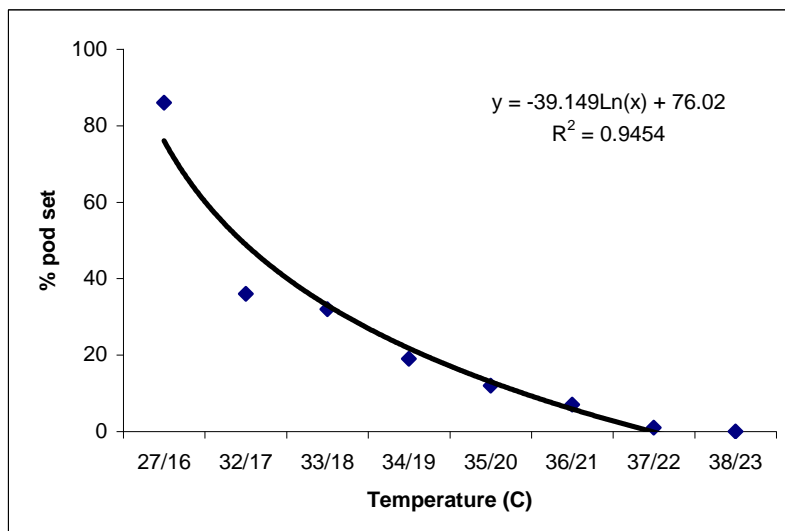$$y = -39.149 \ln(x) + 76.02$$
$$R^2 = 0.9454$$

Fig 7. Relationship between temperature and pod set in the controlled environments (Table 4 mean values % pod set were replotted to predict the reduction by every temperature increase by 1°C using logarithmic regression)
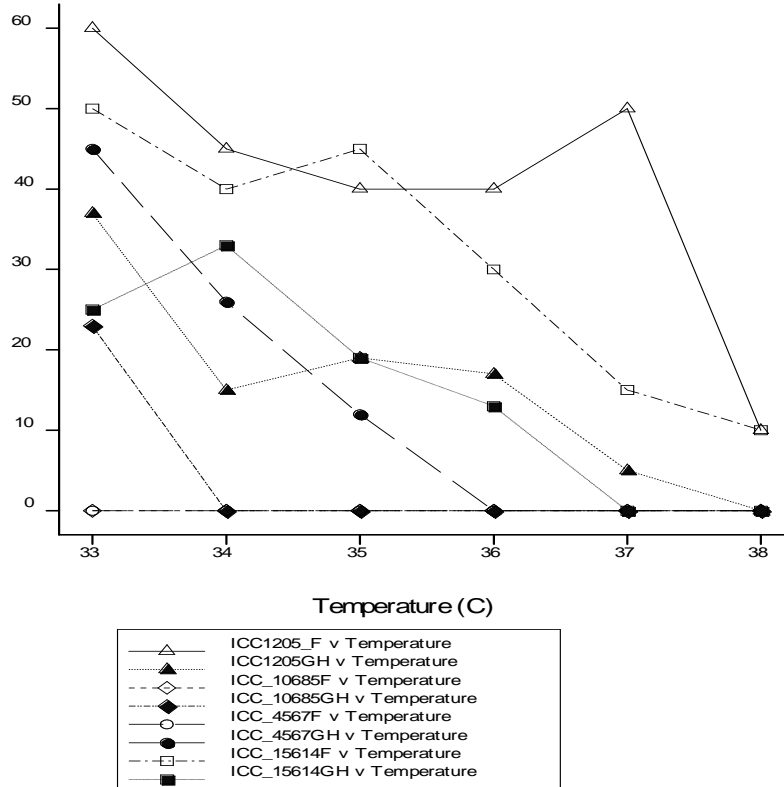

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