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Temperature-stratified screening of chickpea (*Cicer arietinum* L.) genetic resource collections reveals very limited reproductive chilling tolerance compared to its annual wild relatives

J.D. Berger[a,b,*], S. Kumar[c,f], H. Nayyar[c], K.A. Street[d], J.S. Sandhu[e], J.M. Henzell[b], J. Kaur[e], H.C. Clarke[b]

[a] CSIRO Plant Industry, Private Bag No. 5, Wembley, WA 6913, Australia
[b] Centre for Legumes in Mediterranean Agriculture, Faculty of Natural and Agricultural Sciences, The University of Western Australia, 35 Stirling Highway, Crawley, WA 6009, Australia
[c] Department of Botany, Panjab University, Chandigarh 160014, India
[d] International Center for Agricultural Research in the Dry Areas, P.O. Box 466, Aleppo, Syrian Arab Republic
[e] Department of Plant Breeding, Genetics & Biotechnology, Punjab Agricultural University, Ludhiana 141004, India
[f] Centre for Biosciences, School of Basic and Applied Sciences, Central University of Punjab, City Campus, Bathinda, Punjab 151001, India

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ABSTRACT

Low reproductive chilling tolerance in chickpea impairs ovule fertilization, delaying pod set, exposing the crop to terminal drought throughout much of its distribution range. Despite this realization, little progress has been made because of the limited genetic variation available to breeders. To address this issue a wide range of domesticated ($n = 1762$) and wild *Cicer* ($n = 200$) germplasm collected from sites stratified by flowering phase temperature was extensively field evaluated, and compared with *Lupinus angustifolius*, a well-adapted Mediterranean winter annual. Chilling tolerance was estimated by regressing the time interval between pod set and first flower against mean post-anthesis temperature. Field screening was augmented by smaller scale experiments evaluating the effects of contrasting post-anthesis temperature regimes on plant growth and productivity, pollen function and subsequent pod set in temperature-controlled cabinets.

Chickpea was less chilling tolerant than its wild relatives, the flower-pod interval increasing curvilinearly as sites became cooler, with a strong effects between 11 and 16 °C, tailing off after 17.5 °C, but remaining statistically significant. There is little useful variation for chilling tolerance within domesticated chickpea. Small, albeit statistically significant differences in pod set delay in chickpea collected from contrasting flowering phase habitats, were marginal compared to more tolerant species such as *Cicer bijugum*, *Cicer judaicum* and *L. angustifolius*, and to a lesser extent *Cicer reticulatum*, *Cicer pinnatifidum*, and *Cicer echinospermum*. No differences were observed between desi and kabuli types. Field screening identified robust chilling tolerance in a *C. echinospermum* accession that commenced podding earlier, at lower temperatures (10.0 °C), and yielded 5 times more than Rupali, the most productive chickpea. Controlled temperature experiments confirmed that in contrast to chickpea, pollen germination, viability, frequency on the stigma surface and subsequent pod set were unaffected by low post-anthesis temperatures (13/7 °C) in *C. echinospermum* and *L. angustifolius*. Our results indicate that chickpea is even more chilling sensitive than previously thought. Because *C. echinospermum* is inter-fertile with chickpea, it has considerable potential both as a donor of robust chilling tolerance and as an agent for investigating resistance mechanisms.

1. Introduction

Chickpea (*Cicer arietinum* L.) is predominantly grown in the semi-arid tropics under stored soil moisture, or in Mediterranean in-season rainfall systems, either as an autumn- or spring-sown crop, and therefore terminal drought is almost an ubiquitous stress (Khanna-Chopra and Sinha, 1987; Berger and Turner, 2007). Drought escape through appropriate phenology is the principal adaptive strategy in the species (Silim and Saxena, 1993a,b; Siddique et al., 2001; Berger et al., 2004a, 2006), and consequently it is important to minimize delays in the onset of the reproductive phase to allow pod filling to occur while terminal drought stress is relatively low. Low chilling tolerance in chickpea is an example

* Corresponding author at: CSIRO Plant Industry, Private Bag No. 5, Wembley, WA 6913, Australia. Tel.: +61 893336623; fax: +61 893878991.
E-mail address: Jens.Berger@csiro.au (J.D. Berger).

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of this, because it can delay the onset of podding by up to 35 days after flowering in Mediterranean climates in Australia (Berger et al., 2004b).

Reduced pod set under chilling temperatures has long been recognized in chickpea (Savithri et al., 1980; Srinivasan et al., 1998; Croser et al., 2003), and attributed to impaired fertilization resulting from reduced pollen function (Srinivasan et al., 1999; Clarke and Siddique, 2004) and/or ovule viability (Srinivasan et al., 1999; Nayyar et al., 2005a). Despite our understanding of fertilization as the rate limiting step (Srinivasan et al., 1999; Clarke and Siddique, 2004), and the application of pollen selection in breeding (Clarke et al., 2004), improving chickpea chilling tolerance has proved difficult, because of the limited genetic variation available to breeders. Differences between tolerant and susceptible remain small and elusive. For example, while cultivars with consistently improved pollen viability, germination and pollen tube growth, and ovule viability have been identified in both field and controlled temperature studies (i.e. ICCV 88502, 88503), these are still very sensitive to temperature, with low fertilization rates (23–34%) and greatly reduced yield (10–20% of unstressed control values) under cold stress (15 °C day/0 °C night) (Srinivasan et al., 1998, 1999). Similarly, the application of pollen selection – which showed great potential under controlled conditions – has had less impact in the field. While pollen-selected progeny flowered and set pods earlier than their parents, and other previously released cultivars, the thermal time interval between podding and flowering was not reduced (Clarke et al., 2004), suggesting that chilling tolerance gains may be insufficient to cope with temperature fluctuations in the field. This was confirmed by large flower-pod intervals (>65 days) in Rupali and WACPE 2078, products of pollen selection, when grown under low post-anthesis temperatures (~10 °C) in southern Western Australia (Berger et al., 2005).

Limited genetic variation is compounded by practical difficulties in evaluating reproductive chilling tolerance. Field evaluation may be compromised by fluctuating temperature and variable phenology, exposing populations to uneven chilling stress, typically allowing later flowering material to escape. While the use of controlled temperature facilities resolves this problem, it is expensive, and tends to limit the number of genotypes that can be evaluated (i.e. Srinivasan et al. (1999), $n = 6$; Clarke and Siddique (2004), $n = 7$).

Clearly there is a need for more robust chilling tolerance in chickpea. Given the limited genetic diversity currently available to breeders, this will require extensive field screening to accommodate large trials, using a methodology that minimizes chilling escape. To this end, in the present study a wide range of domesticated and wild *Cicer* germplasm was evaluated in field trials in south-western Australia, northern India and Syria, by regressing the time interval between pod set and first flower against mean post-anthesis temperature. Field studies have shown that the flower-pod interval is negatively correlated to post-anthesis temperature below 16 °C (Berger et al., 2004b, 2005), which makes it possible to compare germplasm with variable phenology, flowering across a range of temperatures, because deviations from the common regression line are of interest, rather than any particular absolute value. A focused identification of germplasm strategy (FIGS) approach was used to increase the probability of finding genetic variation in chilling tolerance by modelling flowering temperature (Berger, 2007) at germplasm collection sites (or appropriate research stations, in the case of breeding material). Genotypes from warm and cool habitats were evaluated alongside check cultivars and putatively tolerant material to test the hypothesis that chilling tolerance was subject to environmental selection pressure. Accessions from 5 annual wild *Cicer* species were included in the field screening because earlier work suggested that these had chilling tolerance potential (Berger et al., 2005), but was not extensive enough to draw conclusions about individual species. Contrasting chilling tolerant genotypes identified by field screening were then evaluated under controlled temperature regimes to investigate pollen function and subsequent pod set.

2. Materials and methods

2.1. Field screening for reproductive chilling tolerance

A total of 1762 chickpea accessions, comprising desi, kabuli and pea types from 43 different countries (Table 1) were field-evaluated for reproductive chilling tolerance. Germplasm selection was stratified by temperature of the flowering phase at the collection site (or research station where the material was developed in the case of breeding lines or cultivars) using the methodology outlined in Berger (2007). (The typical flowering phase at each site was defined by modelling, published data and feedback from regional breeders, and then appropriate monthly averages extracted from WorldClim (2010) using DIVA-GIS (Hijmans et al., 2001).) Cool collection sites (14.2 ± 0.06 °C) commonly occurred in northern South Asia, Ethiopia, Portugal, Mexico and Chile; while warm sites (23.2 ± 0.04 °C) were found in southern and central India, continental West Asia, Central Asia and the Caucasus, and East Africa (Fig. 1). Sites with intermediate flowering temperatures (19.8 ± 0.12 °C) occurred in a band from the central Indian subcontinent through to West Asia, eastern and western Europe (Fig. 1). While balanced regional comparisons of cool and warm flowering temperature sites are possible within Europe, East Africa, South and Central Asia, these tend to be separated by considerable distance, except in Ethiopia, Myanmar, and South Asia to a lesser extent (Fig. 1). A wide range of flowering temperatures was available in both kabuli and desi types, albeit that the latter were not formally classified in the ICRISAT collection. While both types were globally distributed, kabulis tended to dominate in WANA and the Americas, whereas desis, including the unclassified ICRISAT collection, were more common in South Asia (Fig. 1). Check lines, advanced breeding material and cultivars were also included in the chilling tolerance screening for comparison purposes (Table 1), and included accessions previously identified as tolerant, including ICCVs 88501 and 88503 (Srinivasan et al., 1998), CTS 60543 (Clarke and Siddique, 2004), Rupali (WACPE 2095) and Sonali (WACPE 2075) (Clarke et al., 2004).

Wild *Cicer* collections are far more limited (Berger et al., 2003), and therefore a stratified sampling strategy as outlined above is infeasible. The germplasm summarized in Table 1 comprises 79–97% of the originally collected accessions in the world collection (Berger et al., 2003), and a large proportion (84–100%) of sub-sampled accessions in all species except for *Cicer judaicum*, where only 5% of the total number of sub-sampled accessions was evaluated. The narrow-leafed lupin (*Lupinus angustifolius*) was included for comparison purposes because drought escape through timely phenology is a key adaptive strategy in the species (Palta et al., 2007). The 25 genotypes listed in Table 1 represent the complete set of Australian cultivars.

The germplasm outlined above was evaluated in separate cool-season field trials conducted over a wide range of sites ($n = 26$) in southwest Australia, northern India and northern Syria over years and/or sowing dates to maximize the likelihood of encountering a wide temperature range during the reproductive phase (Table 2). Indian and Syrian chickpea chilling tolerance trials and the *L. angustifolius* GxE study (details in Berger et al. (in press)), were small and large plot field trials, respectively, whereas all other trials were based on spaced single plants (see Berger et al. (2005) for details of *Cicer* comparisons in 2004). Daily minimum and maximum temperature, dates of flowering and podding were recorded at all sites. (In large plot field trials, dates of 50% flowering and podding were

Table 1
Provenance of germplasm evaluated in screening for reproductive chilling tolerance.

Species	Germplasm evaluated (*n*)	Origin	Chickpea type	Temperature of flowering phase[a] and other categories
C. arietinum	1762	India, 550; Pakistan, 255; Afghanistan, 168; Turkey, 103; Uzbekistan, 100; Ethiopia, 97; Syria, 86; Iran, 74; Mexico, 67; Chile, 22; Iraq, 19; Tajikistan, 20; Azerbaijan, 17; Myanmar, 17; Australia, 16; Portugal, 16; Russian Federation, 16; United States, 13; Turkmenistan, 10; Spain, 8; Ukraine, 8; Armenia, 8; Former Soviet Union, 7; Georgia, 7; Nepal, 7; Israel, 6; Kazakhstan, 6; Sudan, 6; Algeria, 5; Bangladesh, 2; Kyrgyzstan, 5; Bulgaria, 4; Morocco, 4; China, 3; Egypt, 3; Italy, 3; Greece, 2; Moldova, 2; Tunisia, 2; Czech Republic, 1; Jordan, 1; Kenya, 1; Lebanon, 1	Kabuli, 801; Desi, 187; Pea, 65; Unclass[b], 709	Cool (mean = 14.2 °C), 720; Warm (mean = 23.2 °C), 735; Checks, breeding lines and cultivars, 74; Unclass, 233
C. bijugum	45	Turkey, 39; Syria, 5; Iraq, 1		Wild relative of chickpea
C. echinospermum	13	Turkey, 13		Wild relative of chickpea
C. judaicum	35	Syria, 17; Lebanon, 7; Turkey, 4; Israel, 3; Jordan, 3		Wild relative of chickpea
C. pinnatifidum	50	Turkey, 34; Syria, 8; Israel, 6; Lebanon, 1		Wild relative of chickpea
C. reticulatum	57	Turkey, 57		Wild relative of chickpea
L. angustifolius	25	Australia, 25		All Australian cultivars since 1967

[a] Temperature of flowering phase – defined in Berger (2007) by calculating collection site-specific bio-climatic variables.
[b] Unclass – largely unclassified desi types from the ICRISAT collection.

estimated by sub-sampling 10 random plants per plot, whereas in the remaining spaced-plant trials these values were based on a single plant per rep.) The time interval between podding and flowering was calculated, and regressed against immediate post-anthesis temperature (averaged over 20 days) as an index of chilling sensitivity (Berger et al., 2004b) both within and between trial sites. Because chickpea delays pod set at mean immediate post-anthesis temperatures <16 °C (Berger et al., 2004b), genotypes that fall below the regression slope at low temperatures are potentially chilling tolerant.

2.2. Chilling effects on post-anthesis growth (controlled temperature experiment 1)

One kabuli type (Almaz), 2 putatively tolerant (CTS 60543, Sonali) and 1 susceptible (Amethyst) desi chickpeas, and a narrow-leaf lupin (cv. Mandelup) were evaluated under warm (24 °C/15 °C) and chilling (15 °C/7 °C) reproductive phase temperatures, to investigate differences observed in field screening under more controlled conditions. Plants were inoculated with Nodulaid 100® (Group N, chickpea; Group G, lupin) on sowing in potting mix (pH 7) in 91

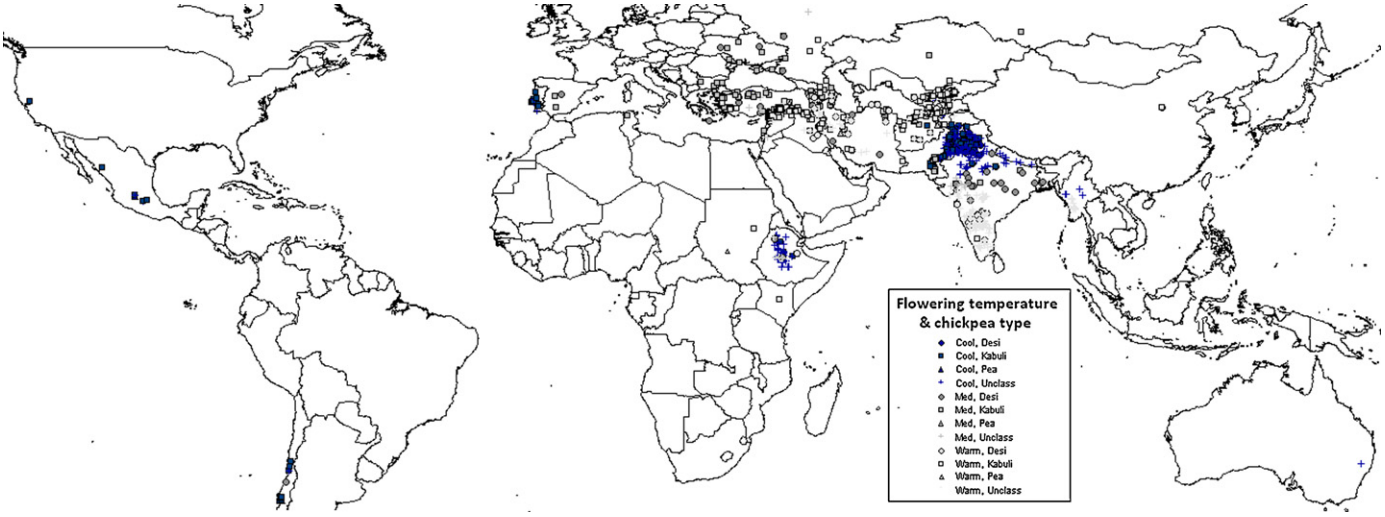

Fig. 1. Collection sites for chickpea germplasm evaluated for reproductive chilling tolerance by field screening. Sites are classified by flowering phase temperature (cool = 14.2 ± 0.06 °C; medium = 19.8 ± 0.12 °C; warm = 23.2 ± 0.04 °C), modelled in Berger (2007) and by chickpea type. Unclassified chickpeas (+) are largely desi types from the ICRISAT collection.

Table 2
Trials summary for the evaluation of wild and domesticated *Cicer* germplasm screened for reproductive chilling tolerance. *L. angustifolius* is included for comparison purposes because drought escape through timely phenology is a key adaptive strategy in the species.

Location[a]	Trial category	Site year	Design[b]	*n*	Species[c]	Sow date	FlowT[d]	RepT < 14[e]
Mt. Barker, WA	Chickpea chill tol	MBC_2008	RCB, *n* = 3	313	*C. arie*, 310; *C. jud*, 2; *C. pin*, 1	25-June	14.1	27
Mt. Barker, WA	Chickpea chill tol	MBC_2009	RCB, *n* = 4	313	*C. arie*, 311; *C. jud*, 2	29-April	13.8	27
Ludhiana, Punjab	Chickpea chill tol	PAU 1 2007	BIB, *n* = 4	619	*C. arie*, 619	19-October	17.7	1
Ludhiana, Punjab	Chickpea chill tol	PAU 2 2007	BIB, *n* = 4	619	*C. arie*, 619	15-November	19.9	0
Tel Hadya, Syria	Chickpea chill tol	TH 2007	BIB, *n* = 3	562	*C. arie*, 562	10-December	20.6	1
Tel Hadya, Syria	Chickpea chill tol	TH 2008	BIB, *n* = 3	483	*C. arie*, 483	28-October	14.8	7
Mt. Barker, WA	Kabuli GxE	MB_ek2004	RCB, *n* = 3	29	*C. arie*, 29	18-May	11.6	46
Floreat, WA	Kabuli GxE	SP_ek2004	RCB, *n* = 3	29	*C. arie*, 29	18-May	13.1	30
Yandanooka, WA	Kabuli GxE	Y_ek2004	RCB, *n* = 3	29	*C. arie*, 29	18-May	13.5	21
Mt. Barker, WA	*Cicer* spp; G x vern	MB_2004	RCB, *n* = 4	148	*C. arie*, 32; *C. bij*, 32; *C. ech*, 12; *C. jud*, 12; *C. pin*, 38; *C. ret*, 22	14-May	13.3	26
Floreat, WA	*Cicer* spp; G x vern	SP_2003	RCB, *n* = 5	218	*C. arie*, 54; *C. bij*, 28; *C. ech*, 14; *C. jud*, 60; *C. pin*, 42; *C. ret*, 20	04-Jul	17.0	6
Floreat, WA	*Cicer* spp; G x vern	SP_2004	RCB, *n* = 4	170	*C. arie*, 32; *C. bij*, 26; *C. ech*, 16; *C. jud*, 30; *C. pin*,36; *C. ret*, 30	14-May	14.8	3
Mt. Barker, WA	Wild *Cicer* spp.	MB_2007	RCB, *n* = 2	171	*C. arie*, 3; *C. bij*, 39; *C. ech*, 6; *C. jud*, 34; *C. pin*,40; *C. ret*, 49	16-May	12.9	34
Mt. Barker, WA	Wild *Cicer* spp.	MB_2008	RCB, *n* = 4	50	*C. arie*, 5; *C. bij*, 44; *C. ech*, 1	06-May	11.1	64
Mt. Barker, WA	Wild *Cicer* spp.	MBE_2010	RCB, *n* = 4	25	*C. arie*, 8; *C. ech*, 5; *C. jud*, 4; *C. pin*,4; *C. ret*, 3; L. angus, 1	26-April	12.0	43
Avondale, WA	Lupin GxE	Av_07	RCB, *n* = 6	25	*L. angus*, 25	08-June	11.9	35
Badgingarra, WA	Lupin GxE	Badg_06	RCB, *n* = 6	25	*L. angus*, 25	15-June	14.4	17
Mt. Barker, WA	Lupin GxE	MB_06	RCB, *n* = 6	25	*L. angus*, 25	20-June	13.4	33
Merredin, WA	Lupin GxE	ME1_05	RCB, *n* = 6	24	*L. angus*, 24	09-May	10.4	57
Merredin, WA	Lupin GxE	ME1_06	RCB, *n* = 6	25	*L. angus*, 25	23-May	12.9	24
Mullewa, WA	Lupin GxE	MU1_05	RCB, *n* = 6	24	*L. angus*, 24	10-May	12.9	50
Mullewa, WA	Lupin GxE	MU2_05	RCB, *n* = 6	24	*L. angus*, 24	10-May	12.9	53
Eradu South, WA	Lupin GxE	VR1_05	RCB, *n* = 6	24	*L. angus*, 24	18-May	13.3	36
Wongan Hills, WA	Lupin GxE	WH1_05	RCB, *n* = 6	24	*L. angus*, 24	24-May	10.3	56
Wongan Hills, WA	Lupin GxE	WH1_06	RCB, *n* = 6	25	*L. angus*, 25	14-June	14.3	17
York, WA	Lupin GxE	York_06	RCB, *n* = 6	25	*L. angus*, 25	16-June	13.5	20

[a] Location: WA, Western Australia.
[b] Design: RCB, randomized complete block and BIB, balanced incomplete block.
[c] Species: *C. arie*, *C. arietinum*; *C. bij*, *C. bijugum*; *C. ech*, *C. echinospermum*; *C. jud*, *C. judaicum*; *C. pin*, *C. pinnatifidum*; *C. ret*, *C. reticulatum*; *L. angus*, *L. angustifolius*.
[d] FlowT: site mean flowering temperature (averaged over 20 days immediately after flowering).
[e] RepT < 14: number of days in the reproductive phase where mean temperatures <14 °C.

plastic pots, and then fertilized with Miracle Grow® monthly. The plants were grown under warm temperatures (24 °C/15 °C, 12/12 h) in growth cabinets until flowering, and then randomly allocated (n = 4 RCBD) to the 2 post-anthesis temperature regimens within 2 weeks of 1st pod set.

Flowers and pods were tagged at 4-day intervals to establish the length of time taken for pod set, and to count the number of flowers and pods. After 23 days the experiment was terminated and plants harvested. Total above ground biomass, seed and pod weight and numbers were measured. Harvest index and seed size were calculated.

2.3. Chilling effects on pollen function (controlled temperature experiment 2)

This experiment investigated pollen biology in response to post-anthesis chilling stress in desi (n = 4; Amethyst (putatively sensitive), ICCV 93929, Sonali, CTS 60543 (putatively tolerant)) and kabuli (n = 3; Almaz, Kaniva, Macarena) chickpeas. In addition, a wild *Cicer echinospermum* accession with chilling tolerance potential (Table 3) was included, while Mandelup lupin used as a tolerant control, as before. Plants were grown under warm conditions in a constant temperature glasshouse (22/18 °C; 12/12 h) until the start of podding, and either left at these temperatures (warm treatment) or subjected to increased chilling stress (13/7 °C) in controlled temperature cabinets. The experiment was conducted as a randomized complete block design (n = 4).

After 1 week acclimatization to the respective warm and cool temperature treatments, plant phenology was assessed daily to select flowers on the day of anthesis (i.e. flower opening). Flowers were excised (n = 4 per plant), and stigmas and pollen grains separated gently. The pollen was bulked and divided to test for viability and *in vitro* germination, as follows. Pollen viability was estimated *in vitro* using the Fluorochromatic Reaction (FCR) test (Heslop-Harrison and Heslop-Harrison, 1970), using a Zeiss AX10 (Carl Zeiss, Thornwood, NY) fluorescence microscope. Stigmal pollen load was estimated by counting pollen grains on the stigma surface of each flower. *In vivo* pollen germination was calculated by proportion (number of stigmas with germinated pollen/total stigma number) during the pollen load examination. Pollen germination *in vitro* was assessed after incubating pollen grains in Brewbaker and Kwack (1963) culture medium at pH 6.0.

The bulked pollen grains were incubated in 50 μl of media at 30 °C for 3 h and germination stopped by adding a drop of Alexander stain to the medium. Pollen was scored as germinated when pollen tube length had reached at least the diameter of the pollen grain. The percentage germination was determined on the basis of a minimum of 100 pollen grains per replicate.

Table 3
Species means for yield components, podding date and temperature from a *Cicer* chilling tolerance trial conducted at Mt. Barker, WA in 2008. Values in parentheses represent the range within species, and can be compared across species using the LSD genotype.

Trait/species	*C. arietinum*[b] (n = 5)	*C. bijugum* (n = 44)	*C. echinospermum*[c] (n = 1)	LSD sp.[d]	LSD genotype
Seed wt g/plant	1.3 (0.3–2.2)	0.8 (0.02–3.0)	10.8	0.6	0.9
Seed no/plant	17.0 (7.2–23.9)	19.8 (1–53.9)	109.7	13.4	18.7
Pod no/plant	24.5 (9.3–33.0)	35.8 (2.4–99.0)	172.1	19.1	26.7
Pod date	8 September (27 August–16 September)	24-September (17 September–5 October)	20-August	6.4	8.9
Podding temp[a] (°C)	10.8 (10.0–11.5)	11.1 (10.5–12.0)	10.0	0.6	0.9

[a] Podding temp: mean temperature averaged over 10 days prior to pod set.
[b] *C. arietinum*: CTS 60543, Rupali, Sonali (all putatively chilling tolerant (Clarke et al., 2004; Clarke and Siddique, 2004)), ICC 7535, ICC 7684.
[c] *C. echinospermum*: selection from ILWC 238.
[d] LSD sp. for comparing species means.

2.4. Chilling effects on pod set (controlled temperature experiment 3)

In order to quantify the effects of chilling stress on pod set, the genotypes listed above (with the exception of Mandelup lupin) were grown in the glasshouse under ambient cool-season temperatures (mean = 14.2 °C) until the onset of flowering. At this point plants (n = 4, RCBD) were transferred into warm (22/16 °C) and cool (13/7 °C) growth cabinets, and the youngest flower on each branch tagged, so that subsequent pods could be identified as having been fertilized in the glasshouse or growth cabinet. After 450 degree days (°d) the plants were removed from the growth cabinet, tagged as before, and the total number of filled and unfilled pods counted. Finally the plants were transferred back to the glasshouse for a 2 week 'recovery' phase (mean temperature = 21.8 °C; 306 °d) to allow pods set in the growth cabinet to finish filling, and to investigate responses to more optimal temperatures after extended chilling. At the end of 2 weeks the total numbers of filled and unfilled pods were counted again, distinguishing between pods set in cabinet or glasshouse.

3. Results

3.1. Field screening for reproductive chilling tolerance

Field screening highlighted the sensitivity of chickpea to chilling temperatures. Fig. 2a is dominated by an apparent curvi-linear response in the flower-pod time interval to immediate post-anthesis temperature in chickpea, contrasted with linear responses in narrow-leafed lupin and some of the wild *Cicer* species, particularly *Cicer bijugum* and *C. judaicum*. Whereas chickpea appears to increasingly delay pod set at temperatures <14 °C, with no evidence of outlying genotypes that can set pods within 2 weeks at temperatures <12 °C, narrow-leafed lupin and some of the wild *Cicer* species appear much more tolerant. Linear regression demonstrated that the apparent curvi-linear responses in Fig. 2a were in fact a series of linear responses to temperature across sites and species, accounting for >85% of variance in field trials in diverse locations in southwest Australia, northern India and Syria. Highly significant 2- and 3-way interactions (P < 0.001) indicated that species' responses to post-anthesis temperature were site-specific, but because of the overlap of observations and fitted curves, these relationships are difficult to discern in Fig. 2a.

Plotting site-specific rates of change of the flower-pod time interval (i.e. the slopes of the fitted curves in Fig. 2a) against immediate post-anthesis temperature calculated individually for each species at each site make these interactions much easier to visualize (Fig. 3). In chickpea, evaluated over the widest temperature range, rates of change in the flower-pod interval increased curvi-linearly as sites became cooler (P < 0.001), with strong effects between 11 and 16 °C, tailing off after 17.5 °C. Nevertheless, even at the warmest sites, temperature-related delays in pod set tend to be significant (P < 0.001); with the warm (19.9 °C) 2nd time of sowing treatment at PAU as the only exception (P = 0.328) among all 14 sites. In the remaining species the rate of change/site temperature relationship was linear, and could be compared with chickpea using linear regression by filtering out the warm site tail-off (>17.5 °C). The combined species by temperature regression analysis accounted for 79.8% of variance and returned significant slope and intercept differences between species (P < 0.001). The intercept for the rate of change/site temperature relationship was most negative in chickpea, indicative of particularly large flower-pod intervals at low temperatures (Fig. 3). Intercept differences between chickpea and *C. bijugum*, *C. judaicum* and *L. angustifolius* were especially marked (P < 0.001), followed by *Cicer reticulatum* (P < 0.007), *Cicer pinnatifidum* (P = 0.053), and finally *C. echinospermum* (P = 0.062). Temperature responses within species were less clear cut. Rates of change in *C. bijugum*, *C. judaicum* and *L. angustifolius* did not vary significantly with immediate post-anthesis temperature, suggesting that the flower-pod interval in these species was insensitive to the temperature range evaluated. Accordingly, slope differences between these species and chickpea were particularly significant (P < 0.001). The *C. reticulatum* response was intermediate, but lower than chickpea (P = 0.02), whereas there were no slope rate differences between *C. pinnatifidum* (P = 0.131), *C. echinospermum* (P = 0.129) and chickpea.

Focussing the flower-pod interval/temperature regression on chickpea facilitated investigation of variation within the crop. Contrasting germplasm collected from cool and warm flowering phase habitats revealed small, but statistically significant differences in chilling tolerance (Fig. 2a). Germplasm from warm habitats was consistently more sensitive, with larger intercepts (P = 0.003), and more negative flower-pod interval/temperature slopes (P < 0.001) at all sites than that from cool areas. Interestingly, the 3-way interaction term was redundant (P = 0.904), implying that there were no site effects on slope differences between germplasm from cool or warm habitats. Further exploring regional effects on warm and cool habitats in a balanced 4-way regression (post-anthesis temperature × habitat type (i.e. warm or cool flowering phases) × region × evaluation site) did little to further define areas of interest for chilling tolerance. (Warm and cool habitats could be compared within and between Europe, East Africa, WANA, Central and South Asia; Fig. 1.) This regression captured marginally more variance (92.1%), and in addition to the trends revealed by the previous analysis, highlighted regional differences in flower-pod interval/temperature slopes (P < 0.001) at all sites, as might be expected, given the range of phenologies sampled across regions. More importantly, it confirmed the lack of interaction in responses of warm or cool habitats within regions, across or within evaluation sites (P = 0.115 and 0.607, respectively).

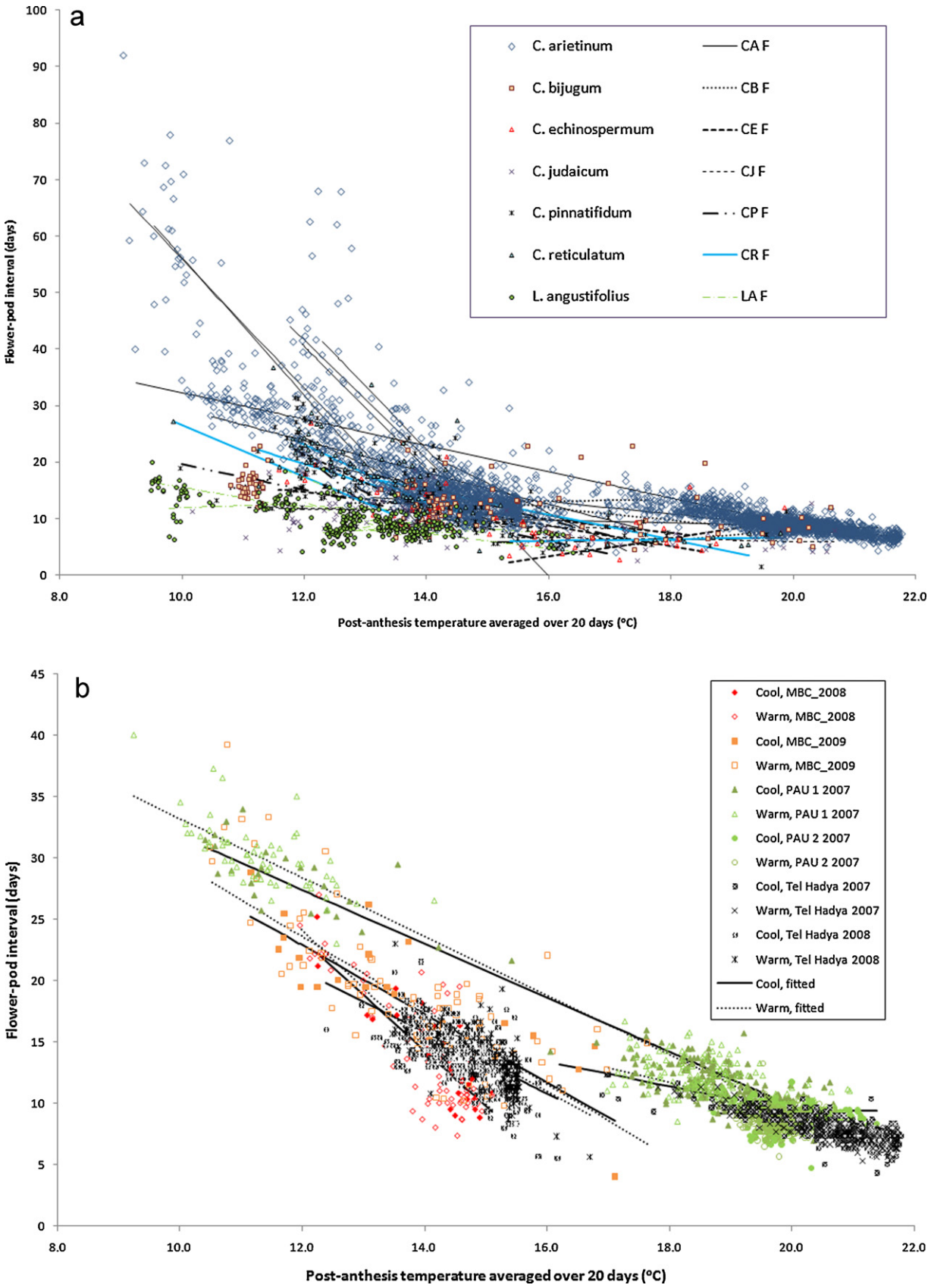

Fig. 2. Flower-pod time interval versus immediate post-anthesis temperature in: (a) narrow-leafed lupin, chickpea and its wild relatives, grown in field trials in Australia, India and Syria. (a) 3-Way linear regression model of post-anthesis temperature by species by trial site accounted for 85.6% of variance, and demonstrated significant slope and intercept differences between species within trial sites ($P < 0.001$), represented by fitted curves presented below. (b) Chickpea classified by contrasting collection site flowering phase temperature (cool = 13.7 °C and warm = 24.1 °C), in the subset of sites where this contrast was balanced. Linear regression accounted for 90.9% of variance, and demonstrated highly significant 2-way interactions between temperature, site and collection site category ($P < 0.003$), but insignificant 3-way interaction ($P = 0.904$).

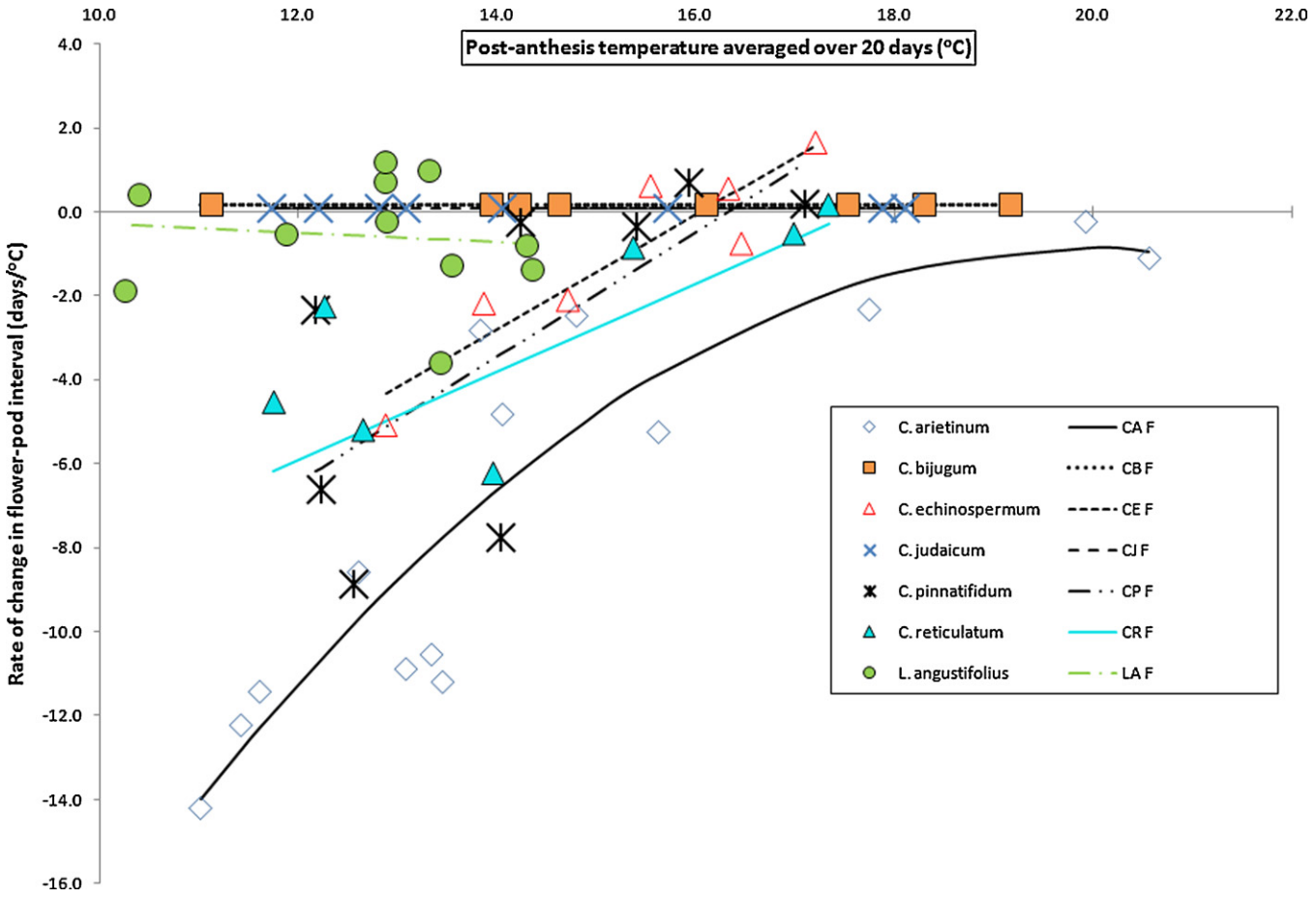

Fig. 3. Site-specific rate of change in flower-pod time interval (slope coefficients from fitted curves presented in Fig. 1) in narrow-leafed lupin, chickpea and its wild relatives evaluated in field trials in southwest Australia, northern India and Syria versus immediate post-anthesis mean temperature calculated individually for species at each site. In chickpea the regression for flower-pod time interval versus post-anthesis temperature was quadratic ($P < 0.001$; adjusted $r^2 = 0.78$), whereas all other curves were linear (see details in text).

Contrasting seed type independently of habitat temperature or origin was much less informative, with no differences in flower-pod interval/temperature slopes between desis and kabulis within or between evaluation sites ($P = 0.504$ and 0.569, respectively).

Data on yield and its components measured during the field screening was generally uninformative, given that pod set occurred at different times and temperatures. Moreover, in the absence of controlled temperature treatments, confounding effects of species or chickpea type were hard to interpret. A *Cicer* chilling tolerance comparison at Mt. Barker in 2008 was the exception, identifying a single *C. echinospermum* accession that set pods very early, at very low temperatures, *and* was considerably more productive than all other genotypes (Table 3). This accession was subsequently tested under controlled conditions (see controlled temperature experiments 2 and 3).

3.2. Chilling effects on post-anthesis growth (controlled temperature experiment 1)

Nested ANOVA effectively summarized differential genotype responses to warm and chilled post-anthesis temperature regimens, highlighting contrasting behaviour between lupins and chickpea, desi and kabuli types, and the lack of residual differences among desi genotypes (Table 4). Significant species by temperature interaction for a range of reproductive traits demonstrated the sensitivity of chickpea to chilling post-anthesis temperatures, and the relative tolerance of the lupin cultivar Mandelup. Thus, for traits such as seed weight per plant, seed and pod number, where chickpea values were typically halved or more in the low temperature treatment, there were no significant differences within Mandelup. The kabuli type Almaz was much more sensitive than the desi varieties, with 0.6–56-fold decreases in these same traits under low post-anthesis temperatures, compared to only 0.1–4-fold in the latter (Table 4). Similarly, the reduction in flower numbers under the chilling treatment was far greater in kabuli than desi types (Table 4).

These trends are clearly demonstrated by principal components analysis, accounting for >92% of variance of the temperature regime by genotype dataset in 2 components (Fig. 4). Lupin was confined to the lower-right quadrant of Fig. 4, clearly separated from chickpea by lower total and vegetative biomass, final pod numbers, smaller flower-pod intervals and higher harvest index, seed size, and proportion of original seed size. Lupin was further distinguished from chickpea by the fact that both temperature treatments plotted close together, reflecting the lack of significant post-anthesis temperature effects in this species. Chickpea was largely characterized by positive PC2 values, temperature treatments clearly separated along PC1 and 2, with particularly strong effects on the kabuli type Almaz, indicative of significant temperature by chickpea type interaction for reproductive traits (listed in bold in Fig. 4). Thus, chickpea-particularly kabuli types, responded to low post-anthesis temperatures by increasing the flower-pod time interval, decreased fecundity (seed and pod numbers and weight), whereas biomass remained unchanged.

Table 4
Temperature regime by genotype interaction *P* values and means for reproductive traits based on orthogonal contrasts of lupin versus chickpea, kabuli versus desi, and remaining genotypic differences among desi types in exp. 1. Means in parentheses are back-transformed values.

Trait *P* values	Lupin versus chickpea	Kabuli versus desi	Within desi
Flower-pod interval (log)	0.006	<0.001	0.700
Tagged flower count (log)	NA	0.047	0.958
Tagged pod count	NA	0.007	0.280
Final pod no (log)	0.002	<0.001	0.450
Seed wt/plant (log)	0.003	<0.001	0.792
Seed no (log)	0.003	<0.001	0.789
Plant biomass (g)	0.105	0.331	0.894

Trait means[a]	Lupin		Kabuli		Desi mean		LSD
Temperature regimen	Warm	Chilled	Warm	Chilled	Warm	Chilled	
Flower-pod interval (log)	0.6 (4)	0.6 (4)	0.9 (8)	1.2 (15)	0.8 (7)	0.9 (8)	0.1
Tagged flower count (log)	NA	1.4 (23)	1.1 (12)	1.5 (31)	1.4 (25)	0.1	
Tagged pod count	NA	18.5	3.3	23.1	18.7	5.3	
Final pod no (log)	0.9 (9)	1.0 (9)	1.5 (33)	0.7 (4)	1.7 (53)	1.5 (30)	0.2
Seed wt/plant (log)	0.2 (1.1)	0.2 (1.5)	−0.1 (1)	−1.8 (0)	0.2 (1.6)	−0.4 (0.4)	0.4
Seed no (log)	1.3 (18)	1.3 (21)	1.4 (23)	0.1 (1)	1.6 (38)	1.2 (17)	0.3
Plant biomass (g)	4.1	6.2	9.0	5.7	10.0	8.9	3.3

[a] Trait mean values are not presented for individual desi genotypes because of the lack of significant differences.

3.3. Chilling effects on pollen competence (controlled temperature experiment 2)

To investigate the role of ovule fertilization in the variable growth responses to chilling temperatures outlined above, an experiment focussing on the effects of these treatments on pollen function was performed. Kabuli–desi differences were examined more rigorously by increasing the number of kabuli accessions ($n = 3$), and a potentially chilling tolerant *C. echinospermum* accession identified in field screening (Table 3) was also included. ANOVA revealed highly significant interaction between species, chickpea type, and post-anthesis temperature for most of the measures of pollen function, while genotypic variation within chickpea types was a relatively minor effect (Table 5). Orthogonal contrasts demonstrate that in general, chickpea was sensitive to low temperatures, kabulis more so than desis, while pollen competence was largely unaffected in *C. echinospermum* and particularly narrow leaf lupin (Table 2). Most measures of pollen competence showed similar responses to chilling temperature. Thus, pollen viability was strongly correlated to both *in vivo* and *in vitro* germination ($r = 0.71$–0.85) and also to the pollen load on the stigmatic surface ($r = 0.82$). Fig. 5 shows the strong relationship between pollen viability and the proportion of flowers with germinating pollen. Under cold conditions (13/7 °C) kabulis tend to have lower pollen viability and *in vivo* germination than desis ($P = 0.001$ and 0.06, respectively), while under warmer temperatures (22/18 °C) these differences disappeared (with the exception of low *in vivo* germination in the desi type Amethyst). (Amethyst had the lowest germination rates under both warm and cold treatments; while CTS 60543 had the highest germination rates of all chickpeas in the cold treatment.)

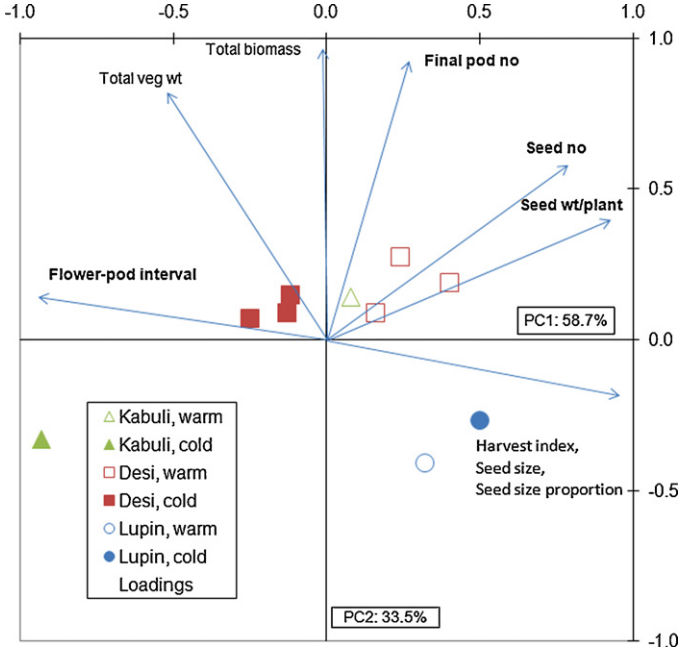

Fig. 4. Principal components analysis of the temperature regime by genotype dataset (exp. 1). Markers represent genotype PC scores categorized by species, chickpea type and post-anthesis temperature regimen (warm: 24 °C/15 °C, empty markers; cold: 15 °C/7 °C; filled markers). Vectors represent variable factor loading coordinates for PC1 and 2. Variables highlighted in bold type have significant temperature by chickpea type interaction (i.e. kabuli versus desi).

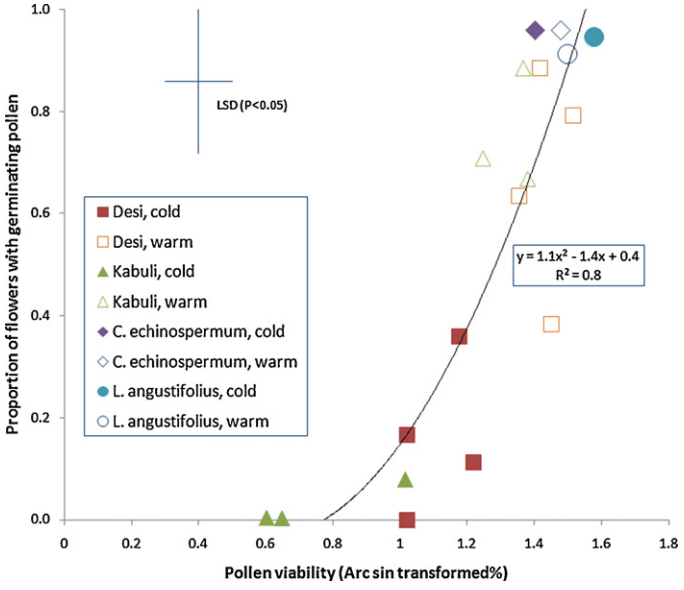

Fig. 5. *In vivo* pollen germination versus *in vitro* viability in narrow-leafed lupin (*L. angustifolius*), domesticated (kabuli and desi) and wild *Cicer* (*C. echinospermum*) grown under warm (22/18 °C; empty markers) and chilling temperatures (13/7 °C; filled markers) (exp. 2).

Table 5
Temperature regime by genotype interaction *P* values and means for reproductive traits based on orthogonal contrasts of lupin versus chickpea, kabuli versus desi, and remaining genotypic differences among desi types in exp. 1. Means in parentheses are back-transformed values.

Trait *P* values	Species × Temp	*C. ech.* versus *L. angus.*	*C. ech.* versus *C. ariet.*	Kabuli versus desi	Within chickpea type
In vitro germination (%)	0.018	0.011	0.008	0.099	0.216
In vivo germination (%)	<0.001	0.867	<0.001	0.060	0.171
In vitro viability (Arc Sin)	<0.001	0.278	0.001	0.001	0.012
Pollen load (log)	0.006	0.586	0.006	0.034	0.004

Trait means	Lupin		Wild *Cicer*[a]		Kabuli mean		Desi mean		LSD
Temperature regimen	Warm	Chilled	Warm	Chilled	Warm	Chilled	Warm	Chilled	
In vitro germination (%)	86	85	91	46	41	22	38	35	19
In vivo germination (%)	91	95	96	96	75	3	67	16	22
In vitro viability (Arc Sin)	1.5 (100)	1.6 (100)	1.5 (99)	1.4 (97)	1.3 (94)	0.8 (47)	1.4 (98)	1.1 (80)	0.2
Pollen load (log)	2.0(98)	2.0 (107)	1.8 (61)	1.9 (89)	1.9 (73)	1.4 (24)	1.8 (65)	1.6 (40)	0.3

[a] Wild *Cicer*: selection from *C. echinospermum* ILWC 238.

Conversely, in both narrow leaf lupin and *C. echinospermum*, neither pollen viability nor *in vivo* germination changed significantly between cold and warm post-anthesis regimens (Fig. 5).

3.4. Chilling effects on pod set (controlled temperature experiment 3)

While the results of experiments 1 and 2 were very consistent, implying that ovule fertilization was likely to be the key step in determining tolerance or susceptibility to reproductive chilling stress, it was important to confirm this hypothesis by studying downstream effects on pod set, particularly in the increased range of genotypes under investigation. By differentiating between pods set from flowers fertilized before or after plants were transferred into growth cabinets it was possible to distinguish the effect of temperature on pod set only (i.e. pods formed from pre-cabinet flowers), and on both fertilization and subsequent pod set (i.e. pods formed from in-cabinet flowers). ANOVA revealed significant ($P = 0.035$) species by temperature interaction for in-cabinet pod set; with 3–5 fold reductions in *C. arietinum* pod set under chilling temperatures, compared with no significant change in *C. echinospermum* (Fig. 6). Nesting cultivars within chickpea type (and species) indicated that while kabuli types appear to be more sensitive than desis (5- and 3-fold reductions, respectively ($P = 0.067$)), there were no genotypic differences within desis or kabuli types ($P = 0.608$). Pod set from pre-cabinet flowers (i.e. fertilized prior to the onset of chilling treatments) was the exact opposite; a 10-fold increase in pod numbers under chilling temperatures in *C. echinospermum*, but no change in *C. arietinum* was responsible for strong species by temperature interaction ($P < 0.001$). Within *C. arietinum* pre-cabinet flower pod set there were no differences in response to post-anthesis temperature between kabuli and desi types ($P = 0.800$), nor between genotypes within these categories ($P = 0.436$).

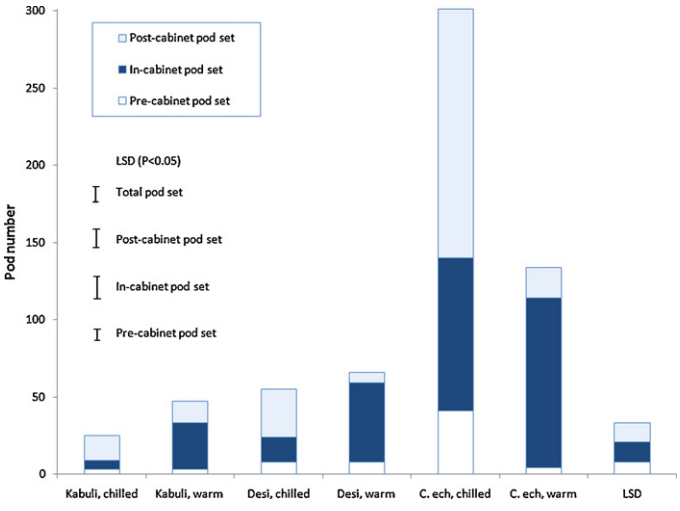

Fig. 6. Pod set in *C. echinospermum* (C. ech), kabuli and desi chickpea types from flowers formed in different environments at different temperatures: (a) pre-cabinet (glasshouse prior to moving to growth cabinet; 14.2 °C), (b) in-cabinet (warm (22/16 °C) or chilled (13/7 °C)) growth cabinets, and (c) post-cabinet (glasshouse for 'recovery phase'; 21.8 °C).

Pod set during the 2 week recovery phase after the 450 °d interval in warm or chilled growth cabinets was largely due to new flowers formed in the glasshouse, and revealed significant temperature interactions between species ($P < 0.001$), desi and kabuli types ($P = 0.006$), and genotypes within chickpea type ($P < 0.001$). Fig. 6 shows that *C. echinospermum* recovery pod set was particularly responsive, with 8-fold increases in plants previously exposed to the chilling treatment compared to those kept under warm post-anthesis temperatures. The desi response trend was similar, but not as marked, with 4-fold increases in the chilled treatment. In contrast, there were no differences in the recovery pod set of kabuli types exposed to chilling or warm post-anthesis temperatures (either individually, or averaged as a group). The significant genotype within chickpea category response ($P < 0.001$) was entirely due to variation within desi types; with strong post-chilling recoveries in ICCV 93929, Amethyst, and particularly Sonali, but not in CTS 60543.

4. Discussion

Our results indicate that chickpea is even more chilling sensitive than previously thought. Although the delay in pod set tails-off at high temperatures, statistically significant delays can be measured at mean flowering temperatures up to 21 °C, considerably higher than the 14–16 °C thresholds reported previously (Berger et al., 2004b, 2005). As a result podding is likely to be delayed in most of the world's chickpea production areas. Flowering phase temperatures were defined for the global distribution of chickpea in Berger (2007), who showed that cool areas (<15.4 °C) were confined to Australia, the northern Indian subcontinent, and parts of the western Mediterranean and the Americas. At a temperature-sensitive threshold of 21 °C the issue becomes global; only production areas in central and southern India, and continental West Asia (eastern Turkey/Syria to Iran and parts of Afghanistan) are likely to escape a delay in pod set. This is important because terminal drought stress is almost ubiquitous across the chickpea distribution range (see references tabulated in Berger and Turner (2007)). Given that escape through early phenology is the primary drought stress adaptive

strategy in chickpea (Silim and Saxena, 1993a,b; Siddique et al., 2001; Berger et al., 2004a, 2006), it is essential to minimize delays in the onset of the reproductive phase. Previous research highlights the significance of even minor phenological delays under terminal drought. In terminally droughted chickpea at Merredin, Western Australia (123 mm seasonal rainfall; postanthesis: 1 mm rainfall, 10% of days with maximum temperature >32 °C), yield was negatively correlated with flowering and podding date (Berger et al., 2004b). Minor delays in podding date (1–3 days) were associated with yield reductions of ~0.2 t/ha among chickpea GxE clusters, a heavy penalty in the context of a 0.7 t/ha mean yield at this site (Berger et al., 2004b). This suggests that even the minor delays in podding recorded in warm sites (>17.5 °C) have the potential to exact considerable yield penalties in chickpea subject to terminal drought, to say nothing of the stronger effects recorded at lower post-anthesis temperatures (<16 °C).

Our field evaluation indicates that there appears to be little useful variation for chilling tolerance within domesticated chickpea. Although germplasm collected from cool areas is statistically more tolerant of sub-optimal temperatures at flowering than that from warm regions, the differences are marginal compared to tolerant species such as *C. bijugum*, *C. judaicum* and *L. angustifolius*, and to a lesser extent *C. reticulatum*, *C. pinnatifidum*, and *C. echinospermum*. This is confirmed by the more detailed controlled temperature experiments, where in general there were no significant differences in chilling tolerance within desi or kabuli types, even when these were selected on the basis of putative contrasting tolerance (i.e. CTS 60543, Sonali, and Amethyst). The apparent increased chilling tolerance of desi compared to kabuli chickpeas observed in experiments using a small number of genotypes (see Nayyar et al. (2007) and our own results) were not supported by our larger scale field screening, suggesting that the germplasm selection in the smaller studies may have been biased.

While our results initially appear to be at odds with the literature, which claims "substantial genetic variation" in chickpea responses to cold stress (Srinivasan et al., 1998, 1999; Clarke and Siddique, 2004), closer scrutiny of this work suggests that the differences reported between tolerant and susceptible are rather minor. Although pollen germination, pollen tube growth, ovule viability and fertilization were consistently higher in the so-called tolerant cultivars (i.e. ICCV 88502, 88503) in both controlled experiments and the field (Srinivasan et al., 1999), cold stress was still very disruptive in these genotypes, leading to 5–10 fold decreases in seed yield (Srinivasan et al., 1998). Similarly, pollen selection has not produced material capable of reducing the flower-pod interval when grown under low post-anthesis temperatures (Clarke et al., 2004; Berger et al., 2005). More recent work reporting considerable chilling tolerance differences among 4 desi genotypes (Kumar et al., 2011), is difficult to put in context because the work was conducted under uncontrolled, relatively warm temperatures, largely >15 °C, and tolerance was confounded with phenology.

Thus we infer that there is very limited variation for chilling tolerance in domesticated chickpea, based both on the published studies using a limited number of genotypes, and our own extensive stratified germplasm screening. This may reflect chickpea's rather unique evolutionary trajectory from Mediterranean winter annual (i.e. as *C. reticulatum*, the wild progenitor (Ladizinsky and Adler, 1976)) to spring-sown Mediterranean crop (Abbo et al., 2003a,b) and its subsequent dissemination to the semi-arid tropics, particularly in South Asia (Redden and Berger, 2007). Throughout its evolution chickpea has consistently avoided cold stress, both in time (by avoiding cold Mediterranean winters through spring-sowing) and space (by moving to warmer southern climates), and appears to have lost adaptive traits that are likely to have existed in its wild progenitors. This argument is consistent with greater vegetative cold tolerance (Singh et al., 1990) and vernalization responses in the wild relatives (Abbo et al., 2002; Berger et al., 2005), but not in domesticated chickpea, and is now strengthened by our discovery of robust reproductive chilling tolerance in a *C. echinospermum* genotype. The significance of this discovery is highlighted by the relative sensitivity of putatively tolerant chickpea genotypes in the same experiments. Thus, while pollen germination, viability and frequency on the stigma surface all declined under chilling stress in chickpea (including the putatively tolerant Sonali and CTS 60543), resulting in reduced pod set, there were few significant differences between temperature treatments in *C. echinospermum*. Moreover, under field evaluation, robust chilling tolerance in *C. echinospermum* facilitated earlier pod set at lower temperatures, leading to >5-fold increases in yield compared to Sonali, the highest yielding chickpea genotype. It is tempting to advance the evolutionary argument that improved chilling tolerance in *C. echinospermum* is an adaptive response to low temperature selection pressure imposed by the winter annual lifecycle in the cool Anatolian plateaux (Berger et al., 2003). To address this hypothesis it will be necessary to evaluate chilling tolerance more extensively within the species, which will necessarily entail more collection, given that the world's genetic resources of *C. echinospermum* currently stand at only 10 original accessions (Berger et al., 2003). Comparative physiological studies of chilling stress responses in *C. echinospermum* and chickpea as related cold-tolerators and avoiders are likely to be particularly interesting. In chickpea, chilling stress has wider ramifications than impaired fertilization alone; having been associated with structural damage to leaves, leading to reduced leaf water and chlorophyll content, impaired carbohydrate metabolism and increased oxidative stress (Nayyar et al., 2005b, 2007; Kumar et al., 2011). This begs the question to what extent these symptoms are expressed in *C. echinospermum* in response to chilling stress.

While our results confirm fertilization as the key bottleneck in chickpea chilling tolerance, supporting earlier work (Savithri et al., 1980; Srinivasan et al., 1999; Clarke and Siddique, 2004), podding responses to cold treatment in *C. echinospermum* raise more questions. While pod set *per se* was unaffected by chilling temperatures in both desi and kabuli chickpeas, as long as fertilization took place under warmer temperatures, there was a 10-fold increase in cold pod set in *C. echinospermum*. This observation is difficult to explain, given that plants were the same age and size, randomly assigned to temperature treatments, and therefore likely to have had a similarly small number of flowers prior to their transfer to warm or chilled growth cabinets. That being the case, this result suggests that pod abortion rates were reduced in the chilling treatment. Recovery from chilling stress may also play an important role in perceived chilling tolerance. While the sensitive kabuli types did not respond after 450 °d exposure to chilling temperatures, *C. echinospermum* and 3 of the 4 desi types bounced back strongly, with 8- and 4-fold increases in 'recovery' pod set in a warm glasshouse (21.8 °C) compared to plants grown under warm post-anthesis temperatures. Indeed, chilled *C. echinospermum* and desi chickpeas set more than 50% of their final pod number during the 306 °d warm 'recovery' period. Verifying and understanding the basis of chilling stimulus to pod set and recovery in *C. echinospermum* is important to give a practical context to this phenomenon. While 45 days of continuous chilling temperatures (i.e. 450 °d at 13/7 °C) are unlikely to occur in any of the world's chickpea production regions, it is possible that positive responses to shorter chilling periods may pay yield dividends.

Our results have important implications for furthering chilling tolerance in chickpea. *C. echinospermum*, a member of the secondary gene pool that readily crosses with domesticated chickpea (Ahmad and Slinkard, 2004) and has been used to improve *Phytophthora* resistance in Australian cultivars (Knights et al., 2008), has considerable potential as both a donor of robust chilling tolerance and as an agent for investigating resistance mechanisms. To this end

recombinant inbred line populations are being established from crosses between *C. echinospermum* and desi (ICCV 93929) and kabuli (Almaz) chickpea to generate hybrids with contrasting chilling tolerance.

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