



## Research article

# Securing reproductive function in mungbean grown under high temperature environment with exogenous application of proline

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## ABSTRACT

Escalating temperatures are adversely impacting the production potential of various cool- and warm-season crops, such as Mungbean, therefore effective strategies are required to improve heat tolerance of various crops. Mungbean, a summer season food legume, is seriously affected at temperatures more than 35/25 °C, especially at its reproductive stage, resulting in pollen infertility to induce loss of flowers and potential pods. Proline (Pro), a well-researched stress-related molecule, has been implicated in determining pollen fertility, but its involvement in affecting reproductive function under heat stress is not reported so far. In the present study, it was hypothesised that depletion of endogenous Pro in reproductive components of the flowers of heat-stressed Mungbean plants might impair the reproductive function. To test this hypothesis, Mungbean genotypes (heat tolerant and heat-sensitive), growing in outdoor environment (32.5/17.5 ± 1 °C mean day/night temperature), until on the onset of flowering (30 days after sowing) were subjected to mild heat stress (MS; 40/28 °C) and high heat stress (HS; 45/33 °C), in the absence or presence of 5 mM proline treatment, applied as soil drenching and foliar spray, 2 days before imposition of heat stress. In MS plants, the endogenous Pro showed a significant increase in leaves, anthers, pollen and ovules, while in SS plants, a marked reduction was noticed. In later case, the activity of proline synthesising enzymes (pyrroline-5-carboxylate synthase and pyrroline-5-carboxylate reductase) declined severely, along with a proline catabolism enzyme (proline dehydrogenase) suggesting disruption in proline metabolism in vegetative and reproductive components. This was associated with considerable decrease in pollen germination, stigma receptivity and ovule viability in heat-stressed plants. Simultaneously, leaf tissue showed high damage to cell membranes, leaf water status, stomatal conductance and cellular respiration. Photosynthetic ability (Chlorophyll, Photo system II function), carbon fixation (RuBisCo activity) and assimilation processes (sucrose synthesis and its hydrolysis) were significantly inhibited, in heat-stressed (HS) plants, which impacted the pod number, pod and seed weight per plant. Pro treatment, especially to HS plants resulted in appreciable increase in its endogenous concentration in vegetative and reproductive parts, which significantly improved the pollen fertility as well as stigma and ovule function. At the same time, stress damage to leaves was reduced significantly, leaf water status and chlorophyll were significantly higher, as a result the carbon fixation and assimilation capacity improved notably to increase the pod set, filled pod number, pod weight and seed weight per plants, suggesting a vital role of proline in enhancing the thermo-tolerance. The effects of Pro treatment were more pronounced in heat-sensitive genotype.

## 1. Introduction

Rising temperatures, globally as well as locally, are becoming a major alarm for agricultural crops cultivated in arid and semi-arid parts of the world (Wahid et al., 2007). Heat stress seriously inhibits the

normal growth and development of the plants to severely impair their production potential (Kaushal et al., 2016). Heat stress accelerates the phenology, to hasten and shorten the reproductive growth, to eventually decrease the potential yields in several crops (Prasad and Jagadish, 2015; Kaushal et al., 2016). Reproductive stage has been

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found to be particularly more sensitive to heat stress, as reported in many crops, such as chickpea (Devasirvatham et al., 2012), lentil (Sita et al., 2017a), Mungbean (Kaur et al., 2015), wheat (Farooq et al., 2011) and sorghum (Prasad et al., 2015). (see Table 4)

Mungbean [*Vigna radiata* (L.) R. Wilczek] is the cheapest source of plant protein, which contains protein ranging from (22–27%), and is the main constituent of a balanced diet. It is also a very good source of thiamin, niacin, vitamin B6, pantothenic acid (vitamin B5), iron and magnesium and consumed as a dietary fiber. Seeds of mungbean are also abundant in phytosterin, coumarin and alkaloids, which help in maintaining physiological metabolism of human beings and animals (Schrawat et al., 2013). The plant is also very useful in improving physical and mental abilities of children due to a high content of proteins, amino acids, polyphenols and oligosaccharides, mungbean exhibits antioxidant and antimicrobial activities (Tang et al., 2014). Mungbean is cultivated in the warmer regions of the world on > 6 million ha, and is one of the most vital pulse crops. It is a short duration (65–90 days) grain legume, and has large adaptability as well as low input requirements (<https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4925713/>; Nair et al., 2012). From a production point of view, the crop ranks second to chickpea among grain legumes, and India is the largest producer of mungbean contributing 54% of world production. The crop ranks first in sowing area, total production and an export amount in the world ((HanumanthaRao et al., 2016). Mungbean is usually grown in summer and autumn in an optimum temperature range between 27 and 30 °C and cultivated in arid and semiarid tropics at altitudes below 2000 m in tropics (Pannu and Singh 1993). Being a warm-season crop, it experiences exposures of above-optimal temperature at the reproductive stage during its normal cultivation season and particularly in late-sown conditions (Sharma et al., 2016). The impacts are larger on late-sown crop and consequently its yield gets constrained significantly because of inhibition of vegetative growth, reproductive failures shown as drop in flowers and pods number, less pod filling and reduced seed size (HanumanthaRao et al., 2016). Heat stress results in considerable damage to pod set in Mungbean, which is associated with reduction in pollen function, as viability, germination, tube growth, stigma receptivity and ovule viability. In general, male reproductive tissue shows more sensitivity to heat stage at all the stages of development, compared to female reproductive tissues (Hedhly, 2011).

Proline is an amino acid, which has been strongly implicated in response to various abiotic stresses (Hayat et al., 2012; Liang et al., 2013; de Freitas et al., 2018), including heat stress (Song et al., 2005; Kaushal et al., 2011). Proline has been suggested to have diverse roles in plants, especially under stress environment, such as generation of turgor, storage of carbon and nitrogen, as scavenger of reactive oxygen species (Smirnoff and Cumbes, 1989), molecular chaperone for maintaining the structure of proteins and membranes, regulation of cytosolic pH, as well as redox status, and as component of the stress indication (Hayat et al., 2012) to influence the adaptive responses. Besides these important roles, its metabolism is reported to be associated to many important pathways such as tricarboxylic acid, pentose phosphate, urea cycles, synthesis of purine and the phenylpropanoid pathway (Kaur and Asthir, 2015), suggesting a critical significance of this molecule. Exogenous proline application has been found to confer protection from abiotic stresses such as salt, metals (Hoque et al., 2007; Ahmed et al., 2010; Hossain and Fujita, 2010; Hasanuzzaman et al., 2014), as well as heat (Kaushal et al., 2011), which has been attributed to reduction in membrane injury, oxidative damage and methylglyoxal, enhancement of antioxidants, leaf water status and photosynthetic function, in these studies.

Recent reports indicate that proline appears to play crucial functions in various aspects of sexual reproduction in angiosperms, but its specific role in reproduction of plants is not fully understood (Biancucci et al., 2015). Proline has been implicated in maintaining pollen vitality and fertility; in pollen grains, it may comprise up to 70% of the free

amino acid pool, as in tomato (Schwacke et al., 1999). Arabidopsis mutants defective in first and rate-limiting step in proline synthesis resulted in abnormal and infertile pollen grains, suggesting that proline is vital for developmental and functional aspects of the pollen (Mattioli et al., 2012). The reproductive organs show high expression of genes, which code for transport and metabolic proteins of the proline pathway (Rentsch et al., 1996; Schwacke et al., 1999). It has been suggested that since proline is established to be a compatible solute, it may protect the macromolecular structures in mature pollen grain (Chiang and Dandekar, 1995; Szekely et al., 2008). The role of proline under stress, especially heat stress, in affecting the reproductive function, has never been investigated, which formed the basis of the present study. We hypothesised that high temperatures may disrupt the proline metabolism in reproductive components of Mungbean, resulting in changes in the proline accumulation, which might contribute reproductive failures. We tested this hypothesis in Mungbean, which is sensitive to heat stress and shows impaired reproductive function due to heat stress (Kaur et al., 2015; Patriyawaty et al., 2018; Reardon and Qaderi, 2017).

## 2. Materials and Methods

### 2.1. Raising of plants

Mungbean seeds (SML 832 (relatively heat tolerant), SML 668 (relatively heat sensitive); short duration; about 70 days; obtained from Punjab Agricultural University, Ludhiana, India) were grown at Panjab University, Chandigarh (30.7333° N, 76.7794° E), India, in earthenware pots having a combination of air-dried soil, sand and farmyard manure in a ratio of 2:1:1 (v/v). The soil had a pH of 7.1, a loam containing 56, 48 and 151 kg ha<sup>-1</sup> of available nitrogen, phosphorous and potassium, respectively. Mungbean seeds were treated with *Rhizobium* sp, and 3 seeds were grown in each pot in the last week of March, which were thinned to two per pot, after emergence.

The plants were raised in natural, outdoor environment conditions in a protected enclosed space (Growth conditions: mean day/night temperature: 32.5/17.5 ± 1 °C, light intensity: about 1500–1700 μmol m<sup>-2</sup> s<sup>-1</sup>, relative humidity; 65–70% Fig. 1) until initiation of reproductive stage (30 days after sowing). Subsequently, one set of the plants was maintained at 35/23 °C (control; under controlled environment; details below) while the others were subjected to heat stress, in a controlled environment. For subjecting the 30-d old plants to heat stress, the temperature was raised by 2 °C each day to bring it to 40/28 °C (as day/night temperature; mild heat stress; MS) and 45/33 °C (as day/night temperature; severe heat stress; HS). Controlled environment had about 500 μmol m<sup>-2</sup> s<sup>-1</sup> light intensity, 65–70% relative humidity. The treatments of control, MS and HS were maintained for 33 days until maturity.

### 2.2. Treatments

In our preliminary experiments, done under the same experimental conditions, as described above, we tested various proline concentrations for their effectiveness. Proline (Pro; Sigma) was applied as 2.5, 5.0, 7.5 and 10 mM concentrations through root-drenching to 30-day old plants, 2 days before imposition of heat stress, and was also foliar-sprayed at the same time. The control plants received distilled water in place of Proline, as root drenching and foliar spray (along with Tween-20[Sigma] as wetting agent), at the same time. The concentration of 5 mM of proline (applied both as root-drenching treatment as well as foliar spray) was found to result in maximum benefits to the yield-traits (pod number, seed number and seed weight plant<sup>-1</sup>) in heat-stressed mungbean plants (data on these preliminary experiments not included). Hence, 5 mM Pro was used in the subsequent experiments.

Pro was applied through root-drenching to 30-day old plants, 2 days before imposition of heat stress, and was also foliar-sprayed (about 200 ml plant<sup>-1</sup>), at the same time using hand-sprayer at 11.00 am.

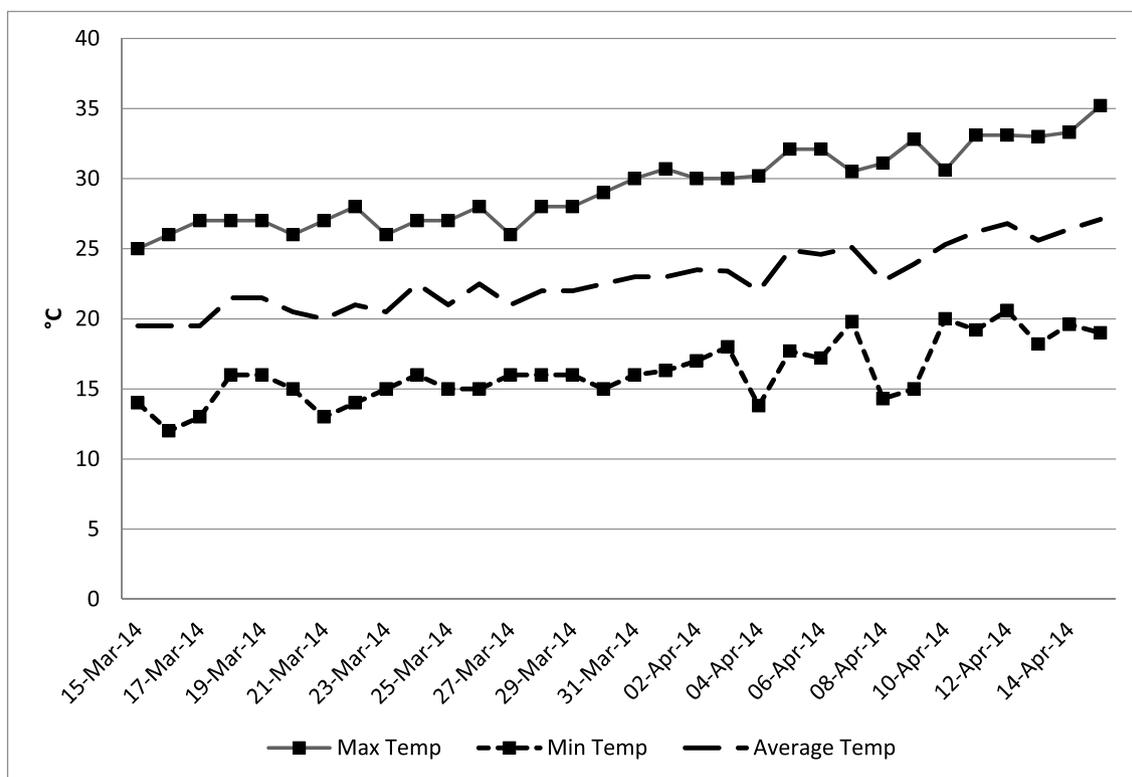


Fig. 1. Temperature profile from sowing until the onset of reproductive phase.

Thus, there were following six treatments:

- Control (no heat stress, no proline application)
- Mild-stress (40/28 °C)
- High-stress 45/33 °C
- Control + 5 mM Pro
- Mild-stress + 5 mM Pro
- High-stress + 5 mM Pro

### 2.3. Analysis of stress injury to leaves

The leaves (from top branches at 2nd and 3rd number) and flowers (for anthers and other tests) were harvested from the control and heat-stressed plants (at 11 am) after 10 days of exposure, and tested for various parameters. The organs were collected from 5 plants per treatment, and replicated thrice (Total 15 plants per treatment).

The leaf tissue was investigated for membrane damage by measuring electrolyte leakage (EL). Initially, the fresh leaves were given a washing using deionized water to eliminate any electrolytes, adhered on surface the leaf tissue was kept in closed glass vials having 10 ml of deionized water, followed by incubation at 25 °C for 24 h on a rotary shaker. Thereafter, the electrical conductivity of the solution (L1) was measured. These samples were then heated at 120 °C for 20 min, in a water bath, followed by after equilibration at 25 °C. Subsequently, the final electrical conductivity (L2) was recorded; electrolyte leakage was defined as  $EL (\%) = (L1/L2) \times 100$  (Lutts et al., 1996).

For measuring the relative leaf water content (RLWC), the leaves (from 2nd-3rd branches) were collected, their initial fresh weight was measured, followed by immersing them for 2 h in distilled water in a petri dish. Thereafter, the tissue was taken out from water and surface-dried using blotting papers, and the turgid weight was measured. The same tissue was dried in the oven at 110 °C for 24 h, and dry weight was measured. RLWC was calculated according to the method of Barrs and Weatherley (1962).

For estimation of total chlorophyll, fresh leaves were collected from

control and heat-stressed plants, and homogenised in 80% acetone to extract the pigments. The extract was measured for chlorophyll at 645 and 663 nm using double-beam spectrophotometer (Arnon, 1949).

Photo-system II (PS II) function was assessed with chlorophyll fluorescence, involving a dark-adapted test with the help of a modulated chlorophyll fluorometer (OS1-FL, Opti-Sciences, Tyngsboro, MA, USA). The instrument's clamps were placed on the top most leaves to maintain a dark environment and to stop the light reaction of photosynthesis, for 45 min. Thereafter, the clamps were fixed to the optic fiber of the instrument, followed by opening of the valves. The instrument was switched-on, and modulated light (695 nm) was given out through the optic fiber into the leaves. PS II was recorded as Fv/Fm ratio (the maximum quantum yield of PSII photochemistry) (Awasthi et al., 2014). Stomatal conductance ( $g_s$ ) of leaves was recorded at 11:00 h, with a portable leaf porometer (model SC1, Decagon Devices, Pullman, WA, USA) (Awasthi et al., 2014). For examining these traits, 4 plants in 3 replication (Total 12 plants per treatment) were tested.

### 2.4. Endogenous proline and metabolising enzymes

For measurement of endogenous proline concentration, the tissues were extracted in 3% sulphosalicylic acid, followed by reaction with acidic ninhydrin reagent (Bates et al., 1973). The enzymes related to proline metabolism were assayed from the tissue samples by homogenizing them in 0.1 M potassium phosphate buffer (pH 7.5) having 1 mM EDTA, 10 mM mercaptoethanol, 5 mM  $MgCl_2$ , 0.6 M KCl and 1% (m/v) polyvinylpyrrolidone, in a pestle and mortar (pre-cooled). The homogenate was centrifuged at 10,000 rpm and 4 °C for 30 min, and the supernatant was de-salted by passing through Sephadex G-100 column, at 4 °C. The active fractions were assayed for enzymes, immediately, as follows.

**Pyrroline-5-carboxylate synthase (P5CS)** activity was assayed by the method described by Filippou et al. (2013). The assay mixture consisted of enzyme extract, buffer (100 mM Tris-HCl; pH 7.2), 25 mM  $MgCl_2$ , 75 mM sodium glutamate, 0.4 mM NADPH, 5 mM ATP. The reaction

rate was assayed as consumption rate of NADPH, which was observed as the reduction in absorption at 340 nm as, a function of time.

Pyroline-5-carboxylate reductase (P5CR) activity was assayed following the method of <https://www.sciencedirect.com/science/article/pii/S0098847203000388> Rena and Splittstoesser (1975). The reaction assay contained enzyme extract, 128  $\mu$ M NADH, 400  $\mu$ M L-P5C and 0.1 M sodium phosphate buffer (pH 7.4) in a total volume of 1 ml. The contents in reference tube were similar except NADH. The reaction commenced with addition of L-P5C, and the enzyme activity was followed for 3 min by assessing the decline in absorbance at 340 nm.

Proline dehydrogenase (PDH) was assayed from the supernatant by adding 0.15 M Na<sub>2</sub> CO<sub>3</sub> buffer (pH 10.3) containing 15 mM proline, 1.5 mM NADP; the NADP reduction was followed at 340 nm in (Ruiz et al., 2002).

## 2.5. Reproductive function

For this purpose, the pollen grains were gathered from the flowers, which opened on the same day, and pooled for testing their viability (<https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Alexander, 1969). Pollen viability was tested by treating them with 0.5% acetocarmine/Alexander stain. The viable pollen grains were chosen on the basis of shape and size (spherical or triangular) and the stain concentration absorbed by the pollen (<https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Kaushal et al., 2013). The observations were recorded in at least 10 microscopic fields.

For measuring, pollen load and pollen germination (*in vivo*), flowers were collected with fully-dehiscent anthers, as well as with and pollen grains on the stigma. The number of pollen grains on stigma surface (pollen load) on the stigma was scored on a 1–5 scale (1 = low number and 5 = high number; <https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Srinivasan et al., 1999). At the same time, the non-germinating and germinating pollen grains on the stigma surface were determined (<https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Kaushal et al., 2013).

For analysis on *in vitro* pollen germination, pollen grains were collected in 3 replications from 5 flowers per genotype, and germinated according to the method of <https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Brewbaker and Kwack (1963). The germination medium comprised of 10% sucrose, 990 mM potassium nitrate (pH 6.5), 1.3 mM calcium nitrate, 1.64 mM boric acid, 812 mM magnesium sulphate. Pollen grains were considered as germinated when the sizes of tube go over the diameter of the pollen grain. The germination (%) was recorded from at least one hundred pollen grains per replicate (<https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Kaushal et al., 2013).

Stigma receptivity was tested using an esterase test, which involved  $\alpha$ -naphthyl acetate as the substrate along with fast blue B, in the azo-coupling reaction (<https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Mattson et al. (1974). Stigmas were collected from the flowers, one day prior to opening of flower; these were dipped in a solution, prepared by dissolving  $\alpha$ -naphthyl acetate and fast blue B in phosphate buffer. The stigmas were kept immersed for 15 min at 37 °C. The surface of stigma developed reddish brown color, the intensity of the color indicated the receptivity, which was rated on a 1–5 scale (1 = lightest color showing low receptivity and 5 = deepest color showing high receptivity) (<https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Kaushal et al., 2013).

To assess, ovule viability, TTC (2, 3, 5-triphenyl-2 H-tetrazolium chloride) reduction test was used. The fresh ovules, extracted from the ovary, one day before anthesis. A drop of TTC solution (0.5% TTC in 1% sucrose solution) was put on the ovules on a clean glass slide. After covering with cover slip, slide was placed in a Petri-dish having moist 2 layers of filter papers, which was further covered with a black paper and incubated in the dark at 25 °C in growth chamber for 15 min. The ovule viability was tested under the microscope based upon the intensity of

red colour developed because of conversion of TTC to formazan, particularly in the central region. The intensity of red colour in the ovules depends upon the oxidising ability of the cells. The red colour intensity (as ovule viability) was rated on a scale of 1–5 (1 indicating lowest colour intensity and 5 indicating colour highest intensity) (<https://www.frontiersin.org/articles/10.3389/fpls.2017.00744/full> Kaushal et al., 2013).

### 2.5.1. Effect of proline on pollen germination (*in vitro*)

Pollen grains collected from the control plants were germinated at varying temperatures (35, 40, 42, 45 °C) (method for germination described above in reproductive function) in a growth medium supplemented with 5 mM Proline. The response was tested as pollen germination (%).

## 2.6. Photosynthetic activity and sucrose metabolism

Photosynthetic activity was studied by measuring RuBisCo activity, as per the method of Wang et al. (1992); the activity was assayed following the procedure of Racker (1962). The leaves were extracted in a pestle and mortar (pre-cooled) in a buffer having 50 mM BTP (pH 7.0), and 3 mM MBT, 1.5% PVPP, 1 mM benzamidine, 1 mM PMSF, 10 mM DTT, 0.5 mM ATP, 1 mM EDTA, 10 mM MgCl<sub>2</sub> and 10 mM NaHCO<sub>3</sub>. The leaf extract was centrifuged for 40 min, at 4 °C, at 10,000 rpm; the supernatant acted as enzyme extract. The enzyme extract was de-salted instantly by passing it through Sephadex G-25 columns (Sigma, St Louis, MO, USA), which had been pre-treated using a buffer solution having 20 mM HEPES–NaOH (pH 7.5), 0.25 mM MgCl<sub>2</sub>, 0.01% 2-mercaptoethanol, 1 mM EDTA, and 0.05% BSA, at 4 °C. The de-salted extract was analysed directly for enzyme activities (Racker, 1962). The assay mixture (1 ml) contained enzyme extract, 1M Tris buffer (pH 7.8), 0.025M RuBP, 0.5% glyceraldehyde-3-phosphate dehydrogenase, 0.025M 3-phosphoglycerate kinase, 0.05%  $\alpha$ -glycerophosphate dehydrogenase-triose phosphate isomerase, 0.1M GSH, 0.2M ATP, 0.006M NADH, 0.5M MgCl<sub>2</sub> and 0.5M KHCO<sub>3</sub>. The oxidation of NADH, which involved the conversion of 3-phosphoglycerate to glycerol-3-phosphate, was measured at 340 nm. The enzyme unit was described as the amount that catalysed the break-up of 1 mM RuBP min<sup>-1</sup>.

Sucrose synthase and acid invertase activity was assayed as per the following procedure. The samples were homogenized in ice-cold 200 mM HEPES/KOH buffer (pH 7.8) containing 1% (w/v) polyvinylpyrrolidone (PVP), 10 mM dithiothreitol (DTT), 3 mM magnesium acetate and 3 mM EDTA Na<sub>2</sub>·2H<sub>2</sub>O and followed by centrifugation (10,000 rpm) for 20 min at 4 °C. The supernatant acted as source of enzyme and protein. The supernatant was de-salted immediately, as above for RuBisCo activity. Sucrose synthase activity (Hawker et al. (1976) and vacuolar acid invertase activity (Nygaard, 1977) were assayed from the de-salted extract immediately.

Sucrose concentration was analysed following the enzymatic method of Jones et al. (1977). The reducing sugars concentration was analysed using DNSA method (Sumner and Howell, 1935).

## 2.7. Yield

For recording observations on yield traits, 10 plants in 3 replications (Total 30 plants per treatment) were examined. The number of pods, seeds and seed weight seed weight (per plant basis) were recorded at maturity in control and stressed plants. No destructive biochemical assay was done on these plants.

The experiment was conducted in partly outdoor (till flower initiation) and controlled environment (for exposure to heat stress). The experiment was carried out two times, over a phase of 2 years, and the observations were nearly similar in both the years, the findings have been presented only for the 2nd year, due to more consistency.

## 2.8. Statistical analysis

The experiments were conducted in a randomised block design (RBD), there were 3 replications of each observation for a trait, the data was subjected to ANOVA, and least significant values (LSD) were measured ( $P < 0.05$ ). For comparing the differences between the mean values, Turkey's post-hoc test was applied.

## 3. Results

The study had following six treatments, as detailed in the Materials and Methods:

- control (no heat stress or Proline),
- Moderate heat stress (MS),
- High heat stress (HS),
- Control + 5 mM Proline,
- Moderate heat stress + 5 mM Proline,
- High heat stress + 5 mM Proline. The leaves and anthers of mungbean plants were tested for various traits, the findings are detailed below.

### 3.1. Proline metabolism in leaves and reproductive components

#### 3.1.1. Endogenous proline concentration

Under MS treatment, Pro accumulation (Fig. 2) in heat-tolerant genotype increased by 3.2, 3.3, 3 and 4.5 fold in leaves, anthers, pollen grains and ovules, respectively, while it was 3.4, 2.5, 2.2 and 3.1 fold, respectively, in heat-sensitive genotype, compared to their respective controls. Under HS treatment, Pro accumulation was inhibited in both the genotypes, and was 2.17, 1.9, 1.7, 2.2 fold in leaves, anthers, pollen grains and ovules of tolerant genotype, whereas it was 1.8, 1.6, 1.1 and 1.9 fold in sensitive genotypes, compared to their particular controls.

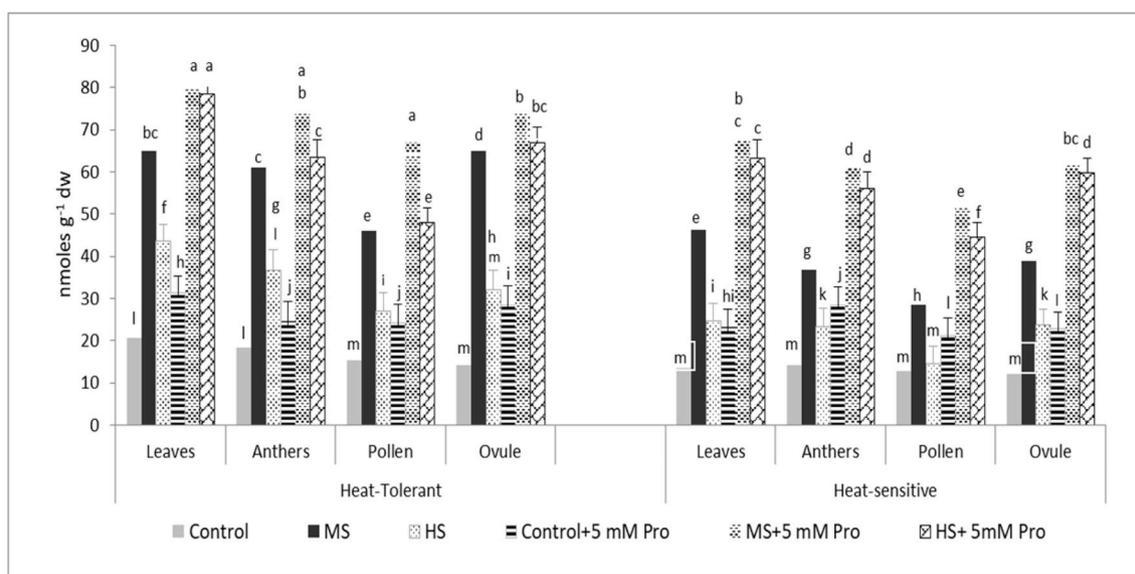
Exogenously applied Pro (5 mM) raised its endogenous concentrations in all the organs, substantially, in both genotypes, in control as well as heat-stressed plants (Fig. 2). The sensitive genotype showed more increase in endogenous Pro in response to its exogenous application. Thus, under HS, tolerant genotypes showed 1.8, 1.7, 1.7 and 2 fold increase in Pro in leaves, anthers, pollen and ovules whereas sensitive genotypes accumulated 2.5, 2.3, 3, 2.5 fold Pro in these organs,

respectively, compared to sensitive plants alone (without Pro treatment). These findings indicated substantial uptake of supplemented proline by all the organs.

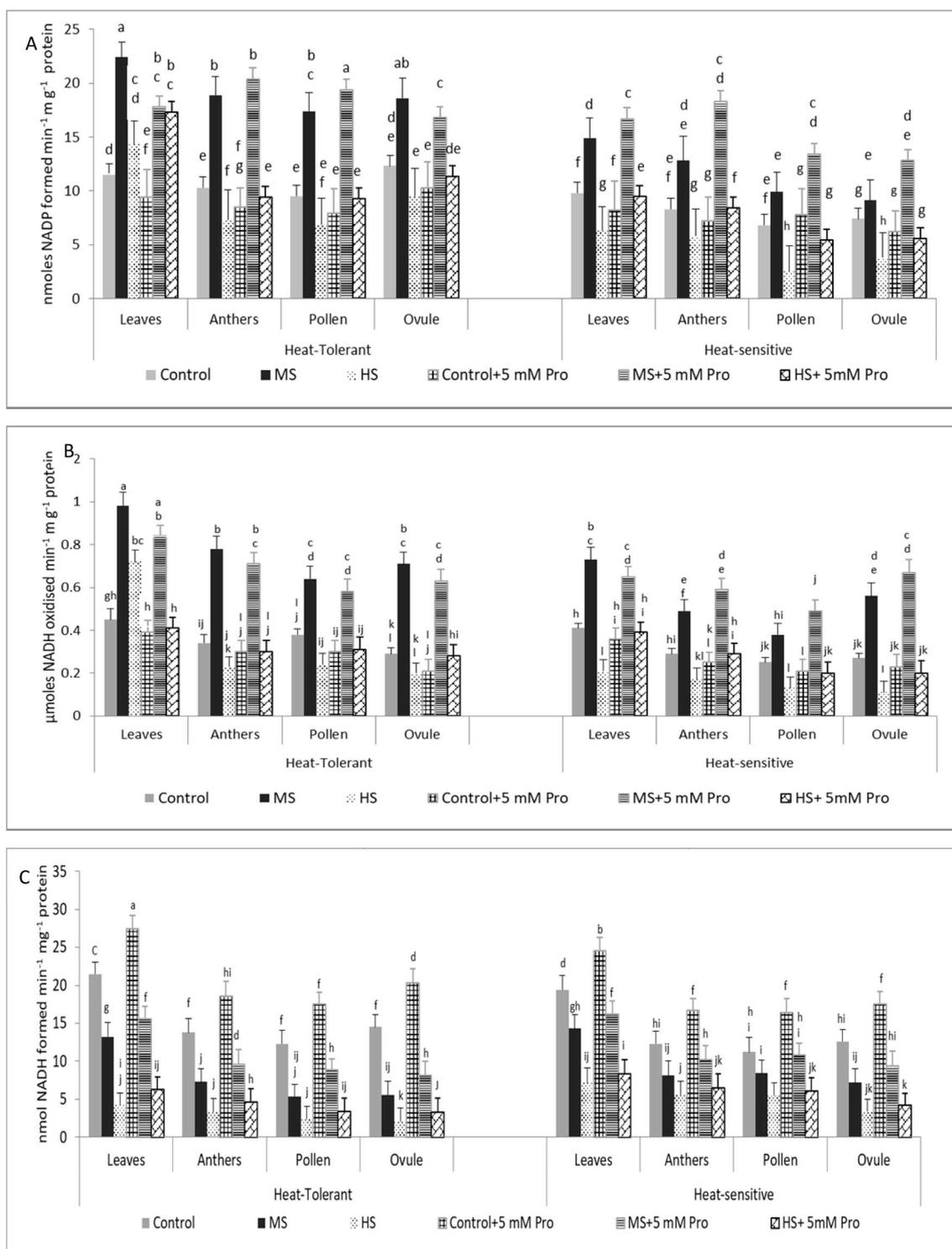
#### 3.1.2. Proline synthesis

Two enzymes involved in synthesis of Pro, **Pyrraline-5-carboxylate synthase** (P5CS) and pyrroline-5-carboxylate reductase (P5CR) were assayed, which showed marked increase in their activities in MS treatment, but not so in HS treatment (Fig. 3). The tolerant genotype showed more expression of their activity levels in all the organs, which correlated with more Pro accumulation. Thus, the activity of P5CS under MS treatment showed increase of 1.9, 1.8, 1.89, and 1.51 fold in leaves, anthers, pollen grains and ovules of tolerant genotype, over their specific controls, whereas the increase in activity was 1.5, 1.5, 1.4 and 1.3 fold, respectively in sensitive genotype, at the same time. In HS treatment, the activity of this enzyme in tolerant genotype increased by 1.2 fold in leaves, while it decreased by 1.4, 1.4 and 1.3 fold in anthers, pollen and ovules, over their respective controls. In sensitive genotype, the activity decreased by 1.5, 1.4, 2.7 and 1.9 fold, in these organs, respectively over the controls. In plants treated with Pro, the activity of P5CS decreased in control as well as MS plants of both the genotypes, over MS plants alone; in contrast, the enzyme activity increased in all the organs of Pro-treated HS plants, in both the genotypes, compared to HS plants alone. The activity showed significantly more increase in sensitive genotype, compared to tolerant genotype, in all the organs (Fig. 3).

Another Pro synthesising enzyme, pyrroline-5-carboxylate reductase (P5CR), also showed marked increase in all the organs of both the genotypes in MS plants alone, over the control, more so in tolerant genotype (Fig. 3). Thus, P5CR activity in MS plants increased over controls by 2.1, 2.3, 1.7 and 2.4 fold in leaves, anthers, pollen grains and ovules, respectively, of tolerant genotype. At the same time, sensitive genotype showed a corresponding increase of 1.8, 1.7, 1.5 and 2 fold, respectively over the controls. In HS plants, P5CR activity increased in leaves of tolerant genotype by 1.6 fold over control while in leaves of sensitive genotype, the activity decreased by 1.9 fold over control. The activity decreased markedly in anthers, pollen grains and ovules, over their respective controls, more so in sensitive genotype. In Pro-treated MS plants of tolerant genotype, the activity decreased over the MS plants alone, whereas sensitive genotype showed increase in



**Fig. 2.** Endogenous proline concentration in heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM; as soil drench and foliar spray, 2 days prior to stress treatment). Values represent mean  $\pm$  SE ( $n = 3$ ). Different letters along with values for a trait indicate significant differences (Tukey's test). LSD ( $P < 0.05$ ) genotype  $\times$  treatment: 5.1.



**Fig. 3.** Pyroline-5-carboxylate synthase (PCS; A), pyroline-5-carboxylate reductase (PCR; B) and proline dehydrogenase (PDH; C) activity in heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM; as soil drench and foliar spray, 2 days prior to stress treatment). Values represent mean  $\pm$  SE (n = 3). Different letters along with values for a trait indicate significant differences (Tukey's test). LSD ( $P < 0.05$ ) genotype x treatment: 2.8 (for PCS), 0.06 (for PCR), 2.2 (for PDH).

activity with Pro. On the other hand, in Pro-treated HS plants, the activity was found to increase in both the genotypes, more so in sensitive genotype, compared to HS plants alone, suggesting differential genotypic response to Pro treatment.

**3.1.3. Proline catabolism**

The activity of Proline catabolising enzyme (Proline dehydrogenase;

PDH; Fig. 3) in MS plants of tolerant genotype decreased over control by 1.6, 2.3, 2.2 and 1.7 fold in leaves, anthers, pollen grains and ovules, respectively. Simultaneously, in sensitive genotype, the enzyme activity showed reduction of 1.3, 1.5, 1.3 and 1.7 fold, respectively over their specific controls. In HS plants, tolerant genotype showed more reduction in PDH activity in leaves (5.1 fold), anthers (2.3 fold), pollen grains (5.1 fold) and ovules (6.9 fold) over control, compared to HS genotype

**Table 1**  
 Various traits related to stress injury in leaves of heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM), as soil drench and foliar spray, 2 days prior to stress treatment). T-tolerant genotype, S-sensitive genotype. Values represent mean  $\pm$  SE (n = 3). Different letters along with values for a trait indicate significant differences (Tukey's test). LSD: least significant difference.

Treatment	Membrane damage (as Electrolyte leakage %)		Cellular viability (OD <sub>485</sub> /50 mg fw)		Chlorophyll (mg/g dw)		Photochemical efficiency (Fv/Fm)		RLWC (%)		Stomatal conductance (gS; mmol m <sup>-2</sup> s <sup>-1</sup> )	
	T	S	T	S	T	S	T	S	T	S	T	S
Control	11.8 $\pm$ 1.32d	11.3 $\pm$ 1.22d	0.36 $\pm$ 0.02b	0.38 $\pm$ 0.02b	14.3 $\pm$ 1.3a	13.6 $\pm$ 1.5a	0.78 $\pm$ 0.09a	0.80 $\pm$ 0.08a	85.6 $\pm$ 2.1a	87.3 $\pm$ 2.3a	407.8 $\pm$ 7.3g	427.3 $\pm$ 6.8f
Moderate heat stress (MS)	21.5 $\pm$ 1.8c	26.4 $\pm$ 1.9b	0.48 $\pm$ 0.024a	0.43 $\pm$ 0.025a	12.1 $\pm$ 1.4b	9.6 $\pm$ 1.5c	0.67 $\pm$ 0.07ab	0.56 $\pm$ 0.06bc	81.3 $\pm$ 2.4ab	71.3 $\pm$ 2.5cd	598.4 $\pm$ 7.1c	670.3 $\pm$ 8.4a
High heat stress (HS)	25.6 $\pm$ 1.8b	30.0 $\pm$ 1.8a	0.23 $\pm$ 0.02cd	0.17 $\pm$ 0.024d	8.9 $\pm$ 1.6c	6.4 $\pm$ 0.036d	0.51 $\pm$ 0.06bc	0.43 $\pm$ 0.06c	64.5 $\pm$ 2.34e	59.4 $\pm$ 2.6de	453.5 $\pm$ 6.8e	331.4 $\pm$ 9.3h
Control + 5 mM Pro	12.4 $\pm$ 1.8e	12.1 $\pm$ 1.22	0.40 $\pm$ 0.024ab	0.40 $\pm$ 0.025ab	13.8 $\pm$ 1.7a	13.1 $\pm$ 0.028a	0.76 $\pm$ 0.07a	0.78 $\pm$ 0.07a	87.3 $\pm$ 2.4a	86.2 $\pm$ 2.2a	409.4 $\pm$ 6.3g	478.3 $\pm$ 10.3e
Moderate heat stress + 5 mM Pro	17.8 $\pm$ 1.5d	19.4 $\pm$ 1.7cd	0.46 $\pm$ 0.026a	0.46 $\pm$ 0.024a	13.1 $\pm$ 1.4a	11.2 $\pm$ 0.028b	0.74 $\pm$ 0.05a	0.67 $\pm$ 0.06ab	83.5 $\pm$ 2.5a	78.3 $\pm$ 2.2bc	591.9 $\pm$ 7.3c	611.3 $\pm$ 9.2b
High heat stress + 5 mM Pro	20.5 $\pm$ 1.4cd	21.3 $\pm$ 1.4c	0.38 $\pm$ 0.025b	0.28 $\pm$ 0.028c	10.9 $\pm$ 1.2b	9.4 $\pm$ 0.027c	0.64 $\pm$ 0.06ab	0.63 $\pm$ 0.07ab	79.9 $\pm$ 2.3bc	71.2 $\pm$ 2.2cd	545.6 $\pm$ 9.5d	421.4 $\pm$ 8.7f
LSD (P < 0.05) Genotype x treatment	3.1		0.07		1.9		0.11		5.4		11.3	

showing corresponding reduction of 2.6, 2.1, 2 and 3.7 fold, respectively. This correlated with higher endogenous Pro in tolerant genotype, than in sensitive genotype. In Pro-treated MS and HS plants, the activity of PDH increased significantly in all the organs of both the genotypes, over MS and HS plant alone, possibly to catabolise the excessive Pro. In both the cases, HT genotype showed significantly more activity than HS genotype, in all the components.

### 3.2. Stress injury to leaves

The stress injury was measured in control and stressed plants on the basis of following traits in leaves:

#### 3.2.1. Membrane damage

It was assessed using electrolyte leakage, which was about 11% in control plants of both heat-tolerant and heat-sensitive genotypes (Table 1). In MS plants, the damage increased to 21.5% in tolerant and 27.3% in sensitive genotype, while in HS plants, the damage was 25.6% and 30%, respectively. In proline (Pro)-treated MS plants, the damage to membranes decreased significantly in both genotypes, more so in sensitive genotype, compared to MS plants alone. In HS plants too, Pro treatment reduced the membrane damage significantly, more in sensitive than tolerant genotype (Table 1).

#### 3.2.2. Cellular viability

In MS plants, cellular viability (CV) was found to increase by 33% in tolerant and 13% in sensitive genotypes, over control plants (Table 1). In contrast, in HS plants, it decreased by 36% in tolerant and 55% in sensitive genotype, over control plants. In Pro-treated HS plants, CV increased by 64–65%, over HS plants alone treatment (Table 1).

#### 3.2.3. Chlorophyll

In MS plants, the chlorophyll (Chl) concentration decreased by 14% in tolerant and 29% in sensitive genotypes, compared to their respective controls (Table 1). It decreased further in HS plants, by 37 and 53% in tolerant and sensitive genotypes, respectively. Pro-treated tolerant genotype showed 13 and 22% improvement in Chl under MS and HS plants, while in sensitive genotype, an increase of 16 and 46% was noticed in MS and HS plants, compared to the heat-stressed plants, growing without Pro (Table 1).

#### 3.2.4. Photochemical efficiency

Compared to control, in MS alone plants treatment, photochemical efficiency (PE) showed 14% reduction in tolerant and 30% in sensitive genotype, while under HS environment, PE decreased by 24% and 46%, respectively (Table 1). PE improved by Pro application to MS plants by 10 and 19% in tolerant and sensitive genotypes, respectively, compared to their particular controls, while in HS plants, Pro treatment resulted in 25 and 46% improvement, respectively (Table 1).

#### 3.2.5. Relative leaf water content

Leaf water status was measured as relative leaf water content (RLWC), which decreased significantly in MS plants of sensitive genotype (71.3%), compared to 87% in its corresponding control, while at the same time, tolerant genotype was significantly less affected (Table 1). Under HS environment, RLWC decreased to 59.4% in sensitive genotype and 64.5% in tolerant genotype. Pro treatment resulted in more improvement in RLWC in sensitive genotype, than in tolerant genotype, both under MS and HS treatment, compared to their respective controls (MS and HS alone) (Table 1).

#### 3.2.6. Stomatal conductance

Stomatal conductance (gS) increased under MS treatment, both in tolerant (47% over control) and sensitive (56% over control) genotypes (Table 1). In HS treatment, tolerant genotype increased gS by 11% over control while it decreased in sensitive genotype by 22%, over control.

**Table 2**

Various traits related to reproductive biology in heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM; as soil drench and foliar spray, 2 days prior to stress treatment). T-tolerant genotype, S-sensitive genotype. Values represent mean  $\pm$  SE (n = 3). Different letters along with values for a trait indicate significant differences (Tukey's test). LSD: least significant difference.

Treatment	Pollen Viability (%)		Pollen Germination (%)		Stigma receptivity (1–5 scale)		Ovule Viability (1–5 scale)	
	T	S	T	S	T	S	T	S
Control	85.6 $\pm$ 2.1b	83.4 $\pm$ 2.1b	83.1 $\pm$ 3.3 ab	86.4 $\pm$ 2.9a	4.8 $\pm$ 0.26a	4.5 $\pm$ 0.28a	4.5 $\pm$ 0.23a	4.3 $\pm$ 0.18 ab
Moderate heat stress (MS)	68.4 $\pm$ 2.5d	53.2 $\pm$ 2.8e	69.5 $\pm$ 2.8b	47.3 $\pm$ 3.2d	3.7 $\pm$ 0.23b	2.8 $\pm$ 0.22d	3.4 $\pm$ 0.21bc	2.9 $\pm$ 0.24c
High heat stress (HS)	43.2 $\pm$ 2.3f	18.4 $\pm$ 2.4g	41.2 $\pm$ 3.3	14.5 $\pm$ 3.7	2.5 $\pm$ 0.28de	1.6 $\pm$ 0.16e	2.9 $\pm$ 0.19c	1.9 $\pm$ 0.22a
Control + 5 mM Pro	90.3 $\pm$ 2.4a	88.5 $\pm$ 2.1a	88.4 $\pm$ 3.5a	90.5 $\pm$ 3.1d	4.6 $\pm$ 0.22a	4.6 $\pm$ 0.24a	4.8 $\pm$ 0.27a	4.6 $\pm$ 0.29a
Moderate heat stress + 5 mM Pro	84.5 $\pm$ 2.8b	77.5 $\pm$ 2.5c	88.4 $\pm$ 3.8a	71.3 $\pm$ 2.9b	4.4 $\pm$ 0.24a	3.9 $\pm$ 0.27b	4.2 $\pm$ 0.27 ab	3.8 $\pm$ 0.29b
High heat stress + 5 mM Pro	61.2 $\pm$ 3.1d	43.6 $\pm$ 2.8e	61.3 $\pm$ 3.9c	41.5 $\pm$ 3.4d	3.3 $\pm$ 0.28c	2.8 $\pm$ 0.21d	3.8 $\pm$ 0.24b	3.3 $\pm$ 0.28c
LSD (P < 0.05) Genotype x treatment	4.8		4.9		0.41		0.40	

Pro-treated HS plants improved gS by 20% in tolerant and 27% in sensitive genotype, compared to their HS plants alone (Table 1).

### 3.3. Reproductive function

#### 3.3.1. Pollen viability

It was about 83–85% in control plants of both the genotypes, but in MS plants, it decreased to 68.4% in tolerant genotype and 53.2% in sensitive genotype (Table 2). In HS plants, pollen viability (PV) declined to 43.2% in tolerant and 18.4% in sensitive genotypes. In the presence of Pro, PV increased significantly, under both stress treatments, as well as in control plants too. Especially, under HS, the PV increased to 61.2% in HT and 43.6% in HS genotypes.

#### 3.3.2. Pollen germination

The control plants had 83% pollen germination (PG) in tolerant and 86.4% in sensitive genotype, which decreased to 69.5 and 47.3%, respectively in MS plants, and further to 41.2 and 14.5%, respectively in HS plants (Table 2). Pro treatment was beneficial and raised the PG in MS treatment to 88% in tolerant and 71.3% in sensitive genotypes. In HS treatment, PG improved to 61.3% in tolerant and 41.5% in sensitive genotype.

#### 3.3.3. Stigma receptivity

The receptivity of stigma (SR) was tested using esterase test, which decreased by 22.9% in tolerant and 38% in sensitive genotype in MS plants whereas in HS plants, it declined by 48 and 73%, respectively, over their specific controls (Table 2). Pro treatment to MS plants improved the SR by 19% in HT and 39% in sensitive genotype, over the MS plants alone. In HS plants, SR improved more (75%) in sensitive genotype than tolerant genotype (32%), compared to HS plants alone.

#### 3.3.4. Ovule viability

The ovules lost their viability by 24% in HT and 32% in HS genotypes in MS treatment, compared to their controls (Table 2). In HS treatment, the reduction was 35 and 56% in tolerant and sensitive genotypes, respectively, over their specific controls. In MS plants, treated with Pro, ovule viability showed improvement of 23% in tolerant and 31% in sensitive genotypes, compared to MS plants alone. In HS plants, Pro treatment improved the viability in HS genotype more (by 73%) than in tolerant genotype (by 31%), over the HS plants alone.

#### 3.3.5. Pro effects on pollen germination at high temperatures

The pollen grains collected from the flowers of the control plants were subjected to varying high temperatures, in the absence or presence of Pro (5 mM) in the growth medium, to test its effectiveness in thermo-protection (Table 3). Pollen grains showed significant reduction in germination at 42 and 45 °C in both the genotypes. At 45 °C, pollen germination was 64% in tolerant genotype and 32.4% in sensitive, compared to around 90% in control (at 35 °C) genotype. Pro treatment markedly improved the germination at 42 and 45 °C in both the

genotypes, the sensitive genotype was more responsive.

### 3.4. Photosynthetic activity and carbon assimilation

#### 3.4.1. RUBISCO activity

Compared to control, the RUBISCO activity in MS plants increased in tolerant genotypes, while it reduced markedly in sensitive genotype (Fig. 4). In HS plants, the enzyme activity decreased significantly in both the genotypes, more so in sensitive genotype (70%), than in tolerant genotype (59%). The activity improved in heat-stressed plants treated with Pro treatment. Thus, in HS plants, the enzyme activity showed 56% improvement in tolerant genotype and 94% in sensitive genotype, compared to HS alone plants.

#### 3.4.2. Sucrose

Sucrose concentration (Fig. 5) increased in MS plants over controls by 19, 23, 24 and 11% in leaves, anthers, pollen and ovules, respectively, in tolerant genotype, whereas in sensitive genotype, under the same environment, sucrose increased by 19% in leaves but decreased by 25, 19, 18% in anthers, pollen grains and ovules, respectively. In HS plants, both the genotypes showed reduction in sucrose, over control plants, more in all the organs of sensitive genotype, compared to tolerant genotype. Pro treatment enhanced the sucrose concentration in heat-stressed plants, which was improved to more extent in sensitive genotype than tolerant genotype. In HS plants of tolerant genotype, Pro treatment resulted in 23, 24, 35 and 29% improvement in leaves, anthers, pollen grains and ovules, respectively, whereas in sensitive genotype, the increase was 32, 44, 50 and 20%, respectively, over the HS alone plants.

#### 3.4.3. Sucrose synthase activity

Relative to the controls, in MS plants of tolerant genotype, the activity of enzyme sucrose synthase (SS; synthesises sucrose; Fig. 5) increased, and was more by 11, 10, 8 and 11% in leaves, anthers, pollen grains and ovules, respectively, while in sensitive genotype, the activity decreased in all the organs. On the other hand, in HS plants, the SS activity decreased in both genotypes, more so, in sensitive genotypes, correlating with the reduction in sucrose concentration. Pro treatment enhanced the enzyme activity in MS and HS plants, compared to their respective controls. In HT genotype, under HS treatment, the activity increased by 22, 19, 14 and 21% in leaves, anthers, pollen grains and ovules, respectively, over the HS plants alone, while in sensitive genotype, a corresponding increase of 21, 24, 26 and 25% was noticed, respectively.

#### 3.4.4. Acid invertases

In MS plants, the activity of acid invertases (vacuolar; convert sucrose into reducing sugars; AI; Fig. 6) increased, more in sensitive genotype, which showed 44, 44, 63 and 64% increase in leaves, anthers, pollen grains and ovules, while tolerant genotype showed corresponding increase of 34, 32, 36 and 38%, respectively, over their

**Table 3**Effect of Proline (5 mM) on *in vitro* pollen germination (%) at varying temperatures, in heat-tolerant and heat-sensitive genotypes (Mean  $\pm$  SE, n = 3).

Treatment	Heat-tolerant genotype				Heat-sensitive genotype			
	35 °C	40 °C	42 °C	45 °C	35 °C	40 °C	42 °C	45 °C
Control(- proline)	91.4 $\pm$ 3.6	88.3 $\pm$ 3.8	79.9 $\pm$ 3.4	63.8 $\pm$ 3.8	89.4 $\pm$ 3.5	86.7 $\pm$ 3.8	58.3 $\pm$ 3.4	32.4 $\pm$ 3.1
Proline (5 mM)	93.2 $\pm$ 3.2	92.3 $\pm$ 3.5	85.9 $\pm$ 3.7	80.4 $\pm$ 3.1	93.4 $\pm$ 3.8	92.1 $\pm$ 3.2	75.6 $\pm$ 3.6	63.43.2
LSD (P < 0.05) Genotype x treatment: 4.2								

specific controls. In Pro-treated MS plants, the AI activity showed significant increase over the MS plants alone. In HS plants, treated with Pro, the AI activity increased in all the organs of both the genotypes, more so in sensitive genotype, than in tolerant genotype.

#### 3.4.5. Reducing sugars

The concentration of reducing sugars (RS) increased in MS and HS plants, compared to control, in an organ-dependent manner (Fig. 6). Thus, in MS plants of tolerant genotype, the RS showed an increase of 25,27,20 and 13% in leaves, anthers, pollen grains and ovules whereas in sensitive genotype, a matching increase of 10, 26,38 and 13%, respectively was noticed. In HS-plants also, tolerant genotype showed more increase in leaves (38%), anthers (37%), pollen grains (45%) and ovules (18%), compared to sensitive genotype, which showed increase of 16, 22, 29 and 8%, respectively, over controls. In Pro-treated plants, RS increased more in MS and HS plants of sensitive genotypes, than in tolerant genotype, over the untreated stressed plants.

#### 3.5. Yield-traits

Compared to control plants, the number of pods per plant in tolerant genotype decreased by 23% and 43% in MS and HS plants, respectively; on the other hand, in sensitive genotype, a corresponding reduction of 40 and 65% occurred (Table 4). With Pro treatment, the pod number showed an improvement of 21 and 30% in MS and HS plants of tolerant genotype, and 38 and 51%, respectively, in sensitive genotype, over MS and HS plants alone.

The pod weight per plant, in tolerant genotype, decreased by 19% and 40% in MS and HS plants while in sensitive genotype, a matching reduction of 27 and 53% was observed, respectively, over their specific controls. Pro treatment resulted in more improvement in sensitive genotype (44% in MS and 45% in HS plants), compared to tolerant

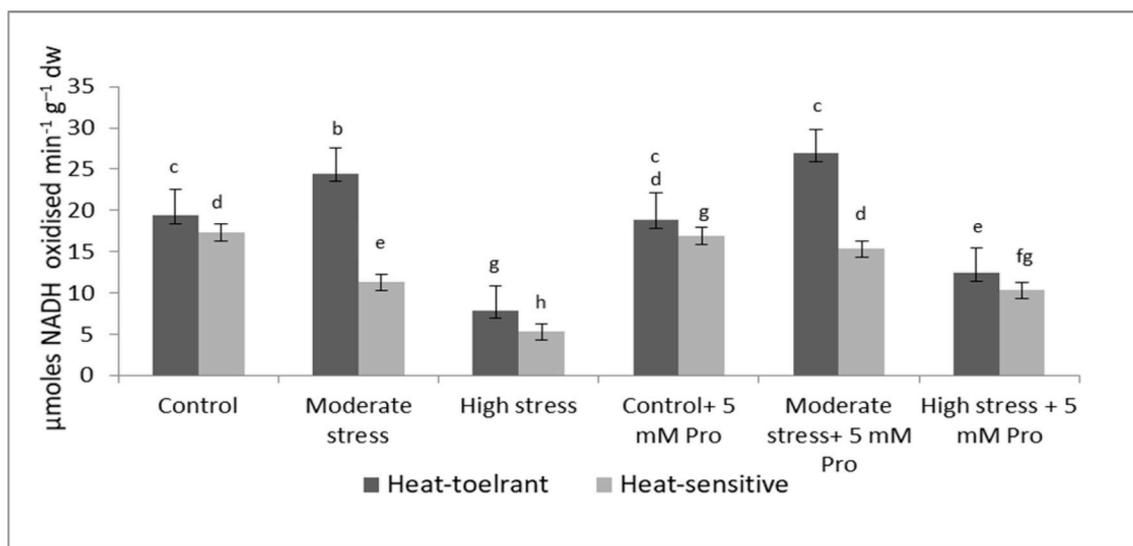
genotype (19% in MS and 43% in HS plants), over MS and HS plants alone.

Seed yield per plant was found to decline by 23.6 and 43.6% in MS and HS plants of tolerant genotype, over their particular controls, while in sensitive genotype, it decreased by 34.6 and 61.5%, respectively. With Pro application to MS plants, the seed yield increased by 23% in tolerant and 38% in sensitive genotype, over MS plants alone. Pro treatment to HS plants resulted in 39% improvement in seed yield of tolerant and 46% in sensitive genotype, over HS plants alone.

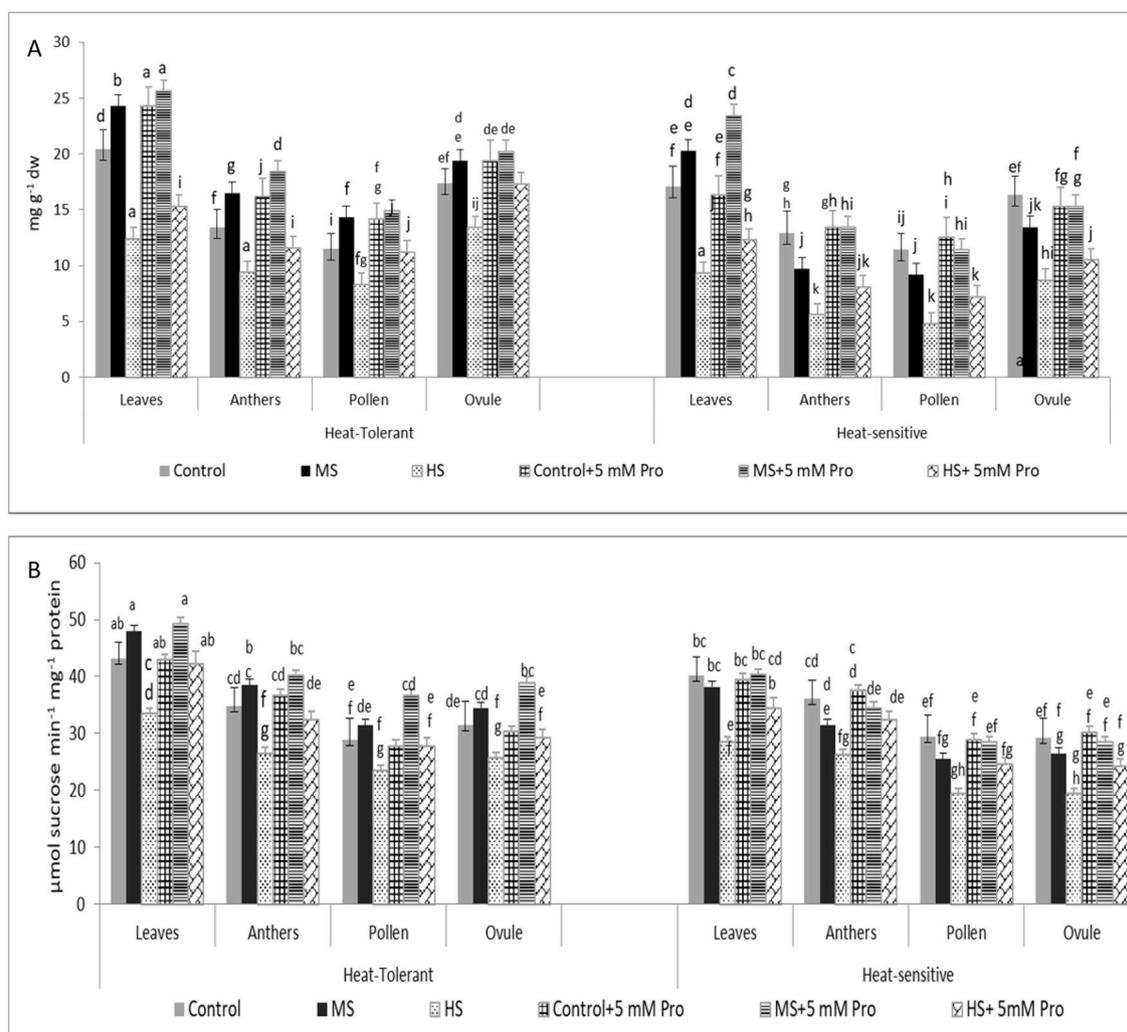
#### 4. Discussion

In the present study, the primary objective was to assess the involvement and role of proline (Pro) in protecting the reproductive function in heat-stressed plants of Mungbean, since, this molecule has been strongly implicated in defence against stresses (Liang et al., 2013), including heat stress (Song et al., 2005; Kaushal et al., 2011), due its multiple roles in stressed plants (Liang et al., 2013). Moreover, Pro has also been suggested to have a role in influencing pollen function (Mattioli et al., 2012), and hence is considered vital for normal sexual reproduction in angiosperms (Biancucci et al., 2015). Our previous studies had indicated a marked reduction in endogenous proline concentration in leaves of severely heat-stressed mungbean plants, which was associated with considerable loss of flowers and pods (Kaur et al., 2015). Based upon these observations, we hypothesised that diminution of proline concentration in heat-stressed mungbean plants might be one of the critical factors inducing damage because of heat stress to leaf as well as reproductive components, to disrupt the reproductive function.

Hence, in the present study, we conducted a detailed analysis on proline metabolism in leaves as well as reproductive components in heat-stressed mungbean plants. Proline (5 mM) was also applied exogenously to heat-stressed plants, to substantiate the depleting



**Fig. 4.** RUBP-carboxylase activity in heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM; as soil drench and foliar spray, 2 days prior to stress treatment). Values represent mean  $\pm$  SE (n = 3). Different letters along with values for a trait indicate significant differences (Tukey's test). LSD (P < 0.05) genotype x treatment: 3.4.



**Fig. 5.** Endogenous sucrose (Suc) concentration and sucrose synthase (SS) activity in heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM; as soil drench and foliar spray, 2 days prior to stress treatment). Values represent mean  $\pm$  SE ( $n = 3$ ). Different letters along with values for a trait indicate significant differences (Tukey's test). LSD ( $P < 0.05$ ) genotype  $\times$  treatment: 2.2 (for Suc), 5.5 (for SS).

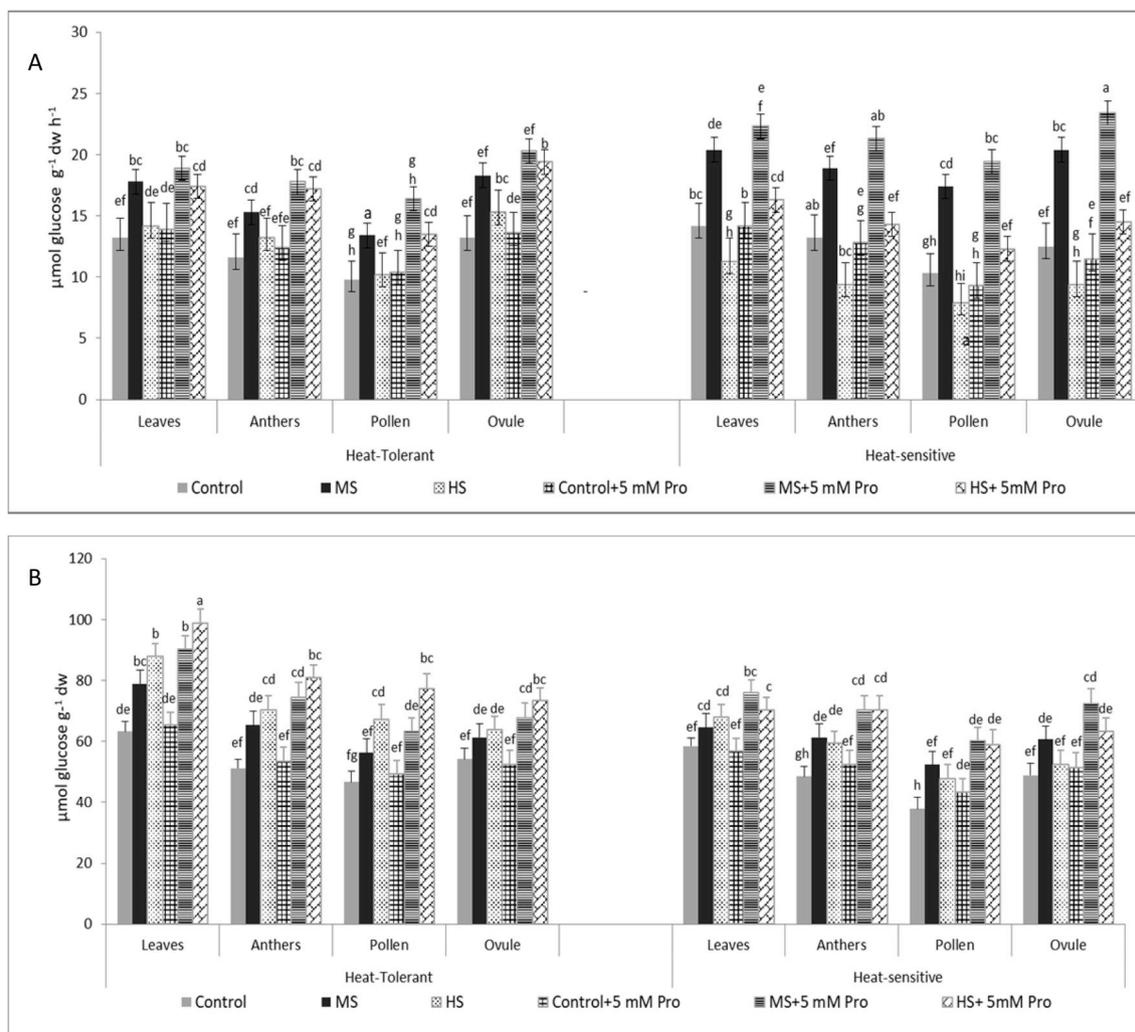
endogenous Pro concentration in vegetative and reproductive tissues, and to test its effectiveness in countering the inhibitory effects of heat stress, especially on reproductive function. We subjected the Mungbean plants to moderate (MS) and high (HS) heat stress, in the absence (control) or presence of Proline (5 mM), applied as root drenching and foliar spray. High temperatures (HS treatment), in the absence of Pro treatment, resulted in marked damage to flowers, pod set, causing substantial decline in number of pods and seeds in heat tolerant as well as heat-sensitive genotypes, which was in accordance with our previous studies (Kaur et al., 2015; Sharma et al., 2016). Exogenous Pro application to heat-stressed plants, especially HS treatment, imparted significant protection to vegetative as well as reproductive components, as indicated by improvement in various functional traits, discussed below.

#### 4.1. Stress injury to leaves

We probed some selective traits related to stress injury caused to vegetative (leaves) and reproductive (flowers) organs in the mungbean plants growing under heat stress environment, without and with Pro treatment. Since, leaves are the primary organs to produce the sucrose and other nutrients to nourish the developing reproductive components, any damage to them because of stress (es) would disrupt the reproductive function. Hence, we tested the leaves for damage to

membranes, photosynthesis, respiration (as cellular oxidising ability) and leaf water status. Membranes' integrity was disrupted considerably in both the genotypes, in high-stressed (HS) plants, more so in HS genotype. Membrane damage occurs due to direct effects (Coria et al., 1998) of high temperature on components of membranes (lipids and proteins), or indirect effects, because of lipid peroxidation (Kaushal et al., 2011), and it has been indicated as a dependable indicator of heat sensitivity (Bita and Gerats, 2013) in chickpea (Kaushal et al., 2013), lentil (Sita et al., 2017a), rice (Sohn and Back, 2007) and wheat (Narayanan et al., 2016).

Cellular respiration was measured using 2,3,5 triphenyl tetrazolium chloride (TTC) reduction assay, which specifies dehydrogenases-related reactions. It increased under MS but decreased under SS environment. The respiration may get affected at above-normal temperatures because of direct impact of high temperature on enzymes (Salvucci and Crafts-Brandner, 2004). The increase or decrease in respiration of leaf tissue in heat stress plants in our case might occur due to stimulation (as in MS plants) or inhibition (as in SS plants) of enzymes by stressful temperature (Kaur et al., 1989), as in wheat (Wang and Nguyen, 1989) and potato (Coria et al., 1998) plants exposed to heat stress. Decreased respiration under high temperature was most likely to occur because of damage to structural well as functional aspects of mitochondria and proteins, and disruption of electron transport rate (Kumar et al., 2013).



**Fig. 6.** Acid invertase (AI; A) activity and reducing sugars (RS; B) concentration in heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM; as soil drench and foliar spray, 2 days prior to stress treatment). Values represent mean ± SE (n = 3). Different letters along with values for a trait indicate significant differences (Tukey's test). LSD (P < 0.05) genotype x treatment: 2.5 (for AI), 5.2 (for RS).

Relative leaf water content (RLWC) indicates the water status; high temperature adversely influences water relations, under water-limited environment (Wahid et al., 2007). RLWC in heat-stressed mungbean plants may decrease as a result of decrease in hydraulic conductivity of the roots, as in tomato (Morales et al., 2003). Our observation in this context is in agreement with those in heat-stressed turfgrass (Jiang and Huang, 2001), Kentucky bluegrass (Liu et al., 2008) and wheat (Sairam et al., 2000) plants.

Stomatal conductance (gS), which impacts RLWC, increased in MS

plants, to maintain leaf temperature (Urban et al., 2017), and can be partly attributed to increased xylem and mesophyll conductance, but decreased in HS plants, more so in sensitive genotype, which correlated with decline in leaf water status in our study, and is similar to observations in lentil (Sita et al., 2017a), Coffee (*Coffea arabica*; Marias et al. 2017). Studies on contrasting genotypes of wheat (Dias et al., 2011) and chickpea (Kaushal et al., 2013) correlated heat tolerance with high stomatal conductance, which (gS) promoted transpirational heat dissipation to maintain leaf temperature. In contrast, loss of

**Table 4**

Various yield-related traits in heat-stressed mungbean plants. Thirty-day old plants were subjected to mild (MS) or high (HS) heat stress, in the absence of presence of proline (5 mM; as soil drench and foliar spray, 2 days prior to stress treatment). T-tolerant genotype, S-sensitive genotype. Values represent mean ± SE (n = 3). Different letters along with values for a trait indicate significant differences (Tukey's test).LSD: least significant difference.

Treatment	Pod number Plant <sup>-1</sup>		Pod yield (g) Plant <sup>-1</sup>		Seed yield (g) plant <sup>-1</sup>	
	T	S	T	S	T	S
Control	12.1 ± 1.8a	11.8 ± 1.4a	7.2 ± 0.82a	6.9 ± 0.67a	5.5 ± 0.65a	5.2 ± 0.56a
Moderate heat stress (MS)	9.3 ± 1.3b	7.1 ± 1.1c	5.8 ± 0.72b	5.0 ± 0.77b	4.2 ± 0.71 ab	3.4 ± 0.68c
High heat stress (HS)	6.9 ± 1.1c	4.1 ± 1.1d	4.3 ± 0.78c	3.2 ± 0.72d	3.1 ± 0.73c	2.0 ± 0.68d
Control+ 5 mM Pro	12.8 ± 1.1a	12.5 ± 1.0a	7.4 ± 0.89a	6.8 ± 0.81a	5.2 ± 0.58a	5.1 ± 0.51a
Moderate heat stress + 5 mM Pro	11.3 ± 1.1a	9.8 ± 1.2b	6.9 ± 0.83a	7.2 ± 0.78a	5.1 ± 0.62a	4.8 ± 0.63a
High heat stress + 5 mM Pro	9.1 ± 1.3b	6.2 ± 1.2cd	6.2 ± 0.85a	4.6 ± 0.81c	4.4 ± 0.64 ab	3.1 ± 0.67c
LSD (P < 0.05) Genotype x treatment	1.7		0.89		0.69	

stomatal conductance has been reported under severe heat stress, as in tobacco (Tan et al., 2011).

#### 4.2. Proline metabolism

Analysis of proline concentration revealed that under MS, its endogenous levels increased manifold in the vegetative and reproductive components in both the genotypes, while under HS, a marked reduction in its concentration, especially in anthers and pollen grains, was noticed. The increase in proline concentration (under MS) or decrease (under HS) correlated with expression of its biosynthesising enzymes Pyrroline-5-carboxylate synthase (P5CS) and Pyrroline-5-carboxylate reductase (PYCR), and match some earlier studies in leaves of water-stressed tall fescue (Man et al., 2011; [*Festuca arundinacea*] and chickpea (Kaur et al., 2017). Proline dehydrogenase (PRODH; a Pro catabolising enzyme) activity decreased under both the stress environments, more under HS, possibly to sustain Pro accumulation, rather than its degradation, and is in agreement with observations in other crop species, such as chickpea (Kaur et al., 2011; Wang et al., 2015). Depletion of Pro in HS plants correlated with increase in stress injury and reduction in yield traits, indicating its involvement in heat sensitivity.

#### 4.3. Carbon fixation and assimilation

Photosynthetic activity was assessed as chlorophyll concentration, chlorophyll fluorescence and RuBisCo activity. The chlorophyll concentration decreased in heat-stressed mungbean plants, as reported in heat-stressed chickpea (Kaushal et al., 2013) and lentil (Sita et al., 2017b). The damage to chlorophyll in heat-stressed plants might be attributed to photo-oxidation of chlorophyll (Guo et al., 2006), and/or because of decrease in biosynthesis of chlorophyll or increased degradation (Tewari and Tripathy, 1998), and/or disorganisation of chloroplasts as a result of photooxidation (Camejo et al., 2006). Leaf symptoms, such as chlorosis, necrosis and bleaching in heat-stressed mungbean plants in our study could be related to photo-oxidative damage or any or all of these reasons (Kaur et al., 2015).

Chlorophyll fluorescence (ChlF) indicates the outcome of excitation energy in chloroplasts, and has been used as marker of photosynthetic efficiency under heat stress (Willits and Peet, 2001). ChlF has shown good correlation with visible leaf damage, electrolyte leakage and water potential of leaves (Larcher, 1995). Its reduction in our studies suggested inhibition of energy transfer mechanisms, hence, photosynthetic ability of heat-stressed mungbean plants, and is similar to the findings in barley (Jedrowski et al., 2014) and wheat (Sharma et al., 2015).

RuBisCO activity expression signifies carboxylation ability; the activity showed increase in MS plants while decreased in HS plants, more so in sensitive genotype. Increase in RuBisCO activity in MS plants of tolerant genotype might have occurred due to enhanced cellular metabolism by temperature (Law et al., 2001), as well as increase in requirement of sugars by the stressed cells (Keunen et al., 2103). Our finding is similar to observations in wheat (Law et al., 2001; Demirevska-Kepova et al., 2005), where tolerant genotype showed more RuBisCO activity than sensitive genotype. In HS plants, the RuBisCO activity might decline due to low stomatal conductance in heat-stressed mungbean plants, and/or as a result of some other reasons, such as reduction in rates of RuBP regeneration, because of disruption of electron transport activity, particularly damage to the oxygen evolving enzymes of photosystem II and activation state of RuBisCO (Salvucci and Crafts-Brandner, 2004).

Sucrose is the principal photoassimilate, which is exported to the developing flowers to support the micro-and mega-sporogenesis (Pressman et al., 2012; Sharma and Nayyar, 2016). Any limitation in sucrose availability to flowers during their development, directly affects them by reducing their size and/or inducing abscission, caused

impaired gametes, thus, leading to no fertilization and abortion of flowers as well as pods (Jain et al., 2008; Awasthi et al., 2014). Heat stress resulted in increase in sucrose in leaves of MS plants, in both the genotypes, in commensuration with increase in RuBisCo activity, while, sucrose concentration decreased in reproductive components (anthers, pollen, ovules) of only HS genotype, which matched the activities of sucrose-synthesising enzymes (sucrose phosphate synthase; SPS) in these organs. In SS plants, more reduction in sucrose concentration in reproductive components, especially in HS genotype, was associated with drastic inhibition in RuBisCo activity in leaves and SPS enzymes in the vegetative and reproductive parts examined. Sucrose starvation can be a major reason for abortion of flowers, pods, reduced pod and seed number, seed size, as reported in lentil (Sita et al., 2017b), chickpea (Kaushal et al., 2013), cotton (Snider et al., 2011), and also might be a primary cause of damage to reproductive function in heat-stressed mungbean plants, in our study. HT mungbean genotype was able to maintain more sucrose concentration, especially in their reproductive components, possibly due to superior heat tolerance of RuBisCo and SPS enzymes. At the same time, the activity of acid invertases (AI), which hydrolyse sucrose, increased under MS environment, more in HS genotypes, which matches the increase in reducing sugars in all the parts tested in our study. In SS plants, HT genotype had more AI activity and reducing sugars, than HS genotype. SPS and AI work together to maintain the concentration of sucrose and reducing sugars at appropriate level in the cells (Nguyen-Quoc and Foyer 2001). Under stress, reducing sugars (hexoses) increase to meet various functional requirements of the cells such as energy, osmoregulation (Anderson and Kohorn, 2001), and thus help the cells to face the adverse environment. In this context, HT genotype maintained sucrose and hexoses' concentrations levels higher than the HS genotype, which possibly contributed towards its superior reproductive function under heat stress environment.

#### 4.4. Reproductive function

Under MS, reproductive function, assessed as pollen viability, pollen germination, stigma receptivity and ovule viability, was inhibited more in sensitive genotype than tolerant genotype, while under HS, both the genotypes showed marked inhibition, more so in former genotype. Damage to these traits by heat stress consequently resulted in flower abortion in mungbean, which is similar to findings in heat-stressed plants in chickpea (Kaushal et al., 2013) and lentil (Bhandari et al., 2016). The reproductive function may be impaired due to direct effects of high temperature on developmental events or indirect effects, such as starvation, because of reduction in availability of sugars and other nutrients to the flowers and their components in our study, and is in conformity with observations in Sorghum (Jain et al., 2008). As a result, pollen function and fertilization processes are impaired to cause abortion of flowers and pods (Prasad and Jagadish, 2015).

#### 4.5. Effects of supplementation of proline

In our study, damage to reproductive function was associated with marked depletion of endogenous proline concentration in anthers, pollen and ovules, which was associated with reduction in the activity of Pro-synthesis enzymes, thus suggesting that appropriate Pro levels might be needed to sustain the reproductive function (Biancucci et al., 2015). In Arabidopsis, the mutants defective in proline-synthesising enzymes produced small, degenerated, unviable pollen grains and were impaired in pollen development, signifying the role for proline in male gametophyte development (Mattioli et al., 2012).

Analysis of anthers, pollen and ovules revealed substantial reduction in endogenous proline concentration in these organs, especially under HS environment, which was associated with sizeable reduction in proline synthesising enzymes, especially in reproductive components. High stress intensity can inhibit proline synthesis and its accumulation

to influence the survival of cells (Liang et al., 2103). Supplementation with Proline to heat-stressed plants resulted in marked increase in its endogenous concentration in leaves, anthers, pollen grains and ovules, both under MS and HS environments, more so in sensitive genotype, which markedly improved the reproductive function, as indicated by increased pollen viability, pollen function, stigma receptivity and ovule viability. We also found considerable enhancement in pollen function with Pro addition in the growth medium under lab environment, which further validated the protective role of Pro for heat-stressed pollen grains. As a result, Pro-treated plants produced more pods and seeds under MS as well as HS, compared to untreated heat-stressed plants. The activity of proline-synthesising enzymes was significantly lower in plants treated with proline, suggesting a feedback inhibition (Zhang et al., 1995). The activity of PDH (catabolises proline into glutamate) increased in proline-treated plants, in contrast to heat-stressed plants, not treated with Pro, which was probably meant to catabolise the excessive proline build-up in the cells (Liang et al., 2013). The sensitive genotype was benefited more, compared to tolerant genotype, which was linked to more depletion of endogenous proline and its synthesising enzymes, in the former genotype.

Moreover, Pro-treated plants maintained high membrane integrity of Mungbean plants under heat stress environments in both the genotypes, more so in sensitive genotype. Pro is reported to stabilise membranes (Matysik et al., 2002) in heat-stressed chickpea (Kaushal et al., 2011), salt-stressed fababean (Gadallah, 1999) and ice plant (Shevyakova et al., 2009). Proline is suggested to scavenge free radicals and buffering cellular redox potential under stress conditions to stabilize sub-cellular structures (e.g., membranes and proteins) (Ashraf and Foolad, 2007). Pro-treated plants showed significantly-improved cellular oxidising ability, which was indicative of protection to respiratory enzymes in heat-stressed mungbean plants, and is in agreement with earlier findings in heat stressed chickpea plants (Kaushal et al., 2011).

RLWC was significantly more in Pro-treated plants, which also improved the stomatal conductance, suggesting that Pro supplementation enhanced the turgor generation and its retention ability, possibly because of its role as osmolyte (Hare and Cress, 1997). In some previous studies too, Pro application to stressed plants increased the leaf water status, as in wheat and barley (Rajagopal and Sinha, 1980), olive (Ben Ahmed et al., 2010) and chickpea (Kaushal et al., 2011).

In heat-stressed plants, growing with Pro, there was significantly lesser damage to chlorophyll and its fluorescence, which might occur due to improved leaf water status, less oxidative damage (Hayat et al., 2012; Liang et al., 2103), and/or protection of enzymes related to chlorophyll synthesis (Paleg et al., 1981). Our observations, in this context, match previous findings in salt-stressed olive plants (Ben Ahmed et al., 2010), heat-stressed chickpea (Kaushal et al., 2011) and salt-stressed rice (Bhusan et al., 2016).

Pro-supplementation resulted in marked improvement in RuBisCO activity in heat-stressed plants, which could be associated with less damage to chloroplasts and photosynthetic pigments (Ben Ahmed et al., 2010). Consequently, these plants also had more sucrose in leaves and flower components under heat stress environment, which was also attributed to enhancement in the activity of sucrose synthesising enzyme in these parts. Enhancement of sucrose concentration might have been a vital factor in improving the reproductive function since previous studies have reported that disruption in sucrose supply to reproductive organs during stress impaired the gamete function (Jain et al., 2008; Snider et al., 2011). We have reported earlier that sucrose supplementation enhances pollen function in heat-stressed pollen grains (Sita et al., 2017b). In this context, sensitive genotype was more responsive to Pro treatment than tolerant genotype. The increase in reducing sugars (hexoses) in vegetative and reproductive parts in Mungbean plants with Pro application could be related to increase in activity of acid invertases.

## 5. Conclusion

Our findings revealed that maintenance of appropriate endogenous proline concentration, in vegetative and reproductive components, is critical in supporting the reproductive function in heat-stressed Mungbean plants. High temperatures deplete the endogenous Pro levels, which probably contribute towards impaired reproductive function, due to disruptions in photosynthetic and photo-assimilation function. Exogenous supplementation of Pro resulted in considerable improvement in various traits linked to leaf and pollen function, which enhanced the pod number and seed yield, in heat-stressed plants. The studies suggest that Pro application to mungbean plants growing in high-temperature stressed environments might impart protection at several levels of organisation of plants.

## Conflicts of interest

The authors declare no conflict of interest.

## Contribution

Manu Priya and Lomeshwar Sharma performed all the experiments and compiled the information. Inderjit Singh, T.S. Bains provided the germplasm. Kadambot H.M. Siddique, Bindumadhava H, Ramkrishnan M. Nair and Harsh Nayyar prepared the final manuscript at different stages and sections.

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