Temperature Mediated Interactions between the Aggressiveness of *Phytophthoradrechsleri* f. sp. *Cajani* and Activity of Defense Enzymes in Pigeonpea

G. SenthilRaja, C. Jyothi Reddy, S. Pande and M. Sharma*
International Crops Research Institute for the Semi-Arid Tropics (ICRISAT), Patancheru–502324, Andhra Pradesh, India
*E-mail: *mamta.sharma@cgiar.org*

ABSTRACT

A pigeonpea field survey was conducted during the year 2010–2013 in the semi-arid tropics of India (Andhra Pradesh, Karnataka and Maharashtra), where the temperature is usually high. Phytophthora blight (PB) of pigeonpea caused by *Phytophthoradrechsleri*f.sp. *cajani* (Pdc) was widespread in all the surveyed areas irrespective of cultivars sown and disease incidence varied between 5–60%.Therefore, the objective of the study was to study the effect of range of temperatures (15–40 °C) on the PB development and on the activity of plant defense enzymes. Under controlled environment studies, we found that disease appeared in all the temperatures with varied incubation period (IP). At higher temperatures (30, 35 and 40 °C), IP was 32, 26 and 18 hours, however at 25, 20 and 15 °C, disease was delayed with an IP of 63, 126 and 225 hours, respectively. To further understand the host, pathogen and environment interactions, biochemical analysis of defense enzymes *viz*., phenylalanine ammonia lyase, peroxidase, polyphenoloxidase and catalase was studied at all temperatures after 0, 24, 48, 72 and 96 hours of inoculation. Activity of defense enzymes in inoculated plants increased progressively at temperatures 25–40 °C during the infection process and then a decline was noticed after the disease progressed. However, at lower temperatures, significant difference was not found in inoculated and uninoculated plants. This study depicts the aggressiveness of Pdcat higher temperatures and will be useful in further strengthening the crop protection practices to mitigate the effect of climate change on plant diseases.

Keywords: Pathogenicity, Temperature,*Phytophthora*, Pigeonpea, Plant defense enzymes

INTRODUCTION

Pigeonpea (*Cajanuscajan* (L.) Millsp.) is one of the most important protein rich grain legume crops being cultivated in the semi-arid tropics of India. India is the largest producer of pigeonpea in the world with a contribution of 75–80 per cent. Despite the large acreage under pigeonpea cultivation in India, the total production and productivity are quite low in most of the pigeonpea growing areas in recent years and emerging diseases are the major constraints to the high yield potential of pigeonpea cultivars. Phytophthora blight, caused by *Phytophthoradrechsleri* Tucker f. sp. *cajani* (Pal *et al*., 1970; Kannaiyan *et al*., 1980), has gained importance in recent years and is an emerging threat to pigeonpea production due to changes in the climatic pattern and erratic rainfall

distribution (Sharma *et al*., 2006; Pande *et al*., 2011). Based on the Intergovernmental Panel on Climate Change's (IPCC) fourth assessment report, changes in global mean temperature of 0.6 – 4°C, in combination with changes in precipitation and an increased frequency of extreme weather events, is likely to occur until 2100 (IPCC, 2007). Even if temperature change is well within the limits of current climatic variability, a modest warming can cause significant increase in heat sums above a critical temperature threshold to affect crop physiology and resistance to disease (Chakraborty *et al*., 2000). In addition, changes in temperature, precipitation and the frequency of extreme events will also influence disease epidemics (Pande and Sharma, 2010; Chakraborty *et al*., 2000; Eastburn *et al*., 2011).

Moisture and temperature are factors known to be of critical importance in governing the infectivity of various *Phytophthora* spp. to different hosts (Tooley *et al*., 2009; Becktell *et al*., 2005; Duniway, 1983; Englander *et al*., 2006; Grove and Boal, 1991; Maziero *et al*., 2009; Timmer *et al*., 2000). Generally, pigeonpea seedlings become vulnerable to its hemibiotrophic pathogen, Pdc as soon as they emerge in the field. This disease is favoured by a temperature of 20–30 °C and humidity of ≥80% (Pande *et al*., 2011). *Phytophthoraramorum* inoculated rhododendron plants expressed the disease over the entire range of temperatures tested (10 to 31 °C), although extent of disease was minor at the temperature extremes (Tooley *et al*., 2009). Similarly, Granke and Hausbek (2010) reported that *P. capsici* is capable of infecting cucumbers over a wide range of temperature (10–35 °C) conditions.

Many plant enzymes are involved in defense reactions against plant pathogens and other abiotic stresses (van Loon *et al*., 2006; Sharma *et al*., 2012). However, there appears to be a common phenomenon that, increase in temperature inhibiting the disease resistance including hyper sensitive response (HR) and modulates the plant defense responses against microbial invasion (Sutherst *et al*., 2011; Zhu *et al*., 2010; Wang *et al*., 2009).Therefore, determining activities of plant defense enzymes against Pdc infection in pigeonpea after exposure to a range of temperatures was an important aspect of the present experiment. Hence, in the present investigation we show the aggressiveness of Pdc as well as activity of four defense related enzymes such as, phenylalanine ammonia lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) and catalase (CAT) in the pigeonpea plants challenged with Pdc after exposure to different range of temperatures.

MATERIALS AND METHODS

EXPERIMENTAL CONDITIONS

The pathogenicity study was conducted within a controlled environment research facility (Greenhouses and CONVIRON growth chambers) located at the International Crops Research Institute for the Semi-Arid Tropics (ICRISAT),

Patancheru, India where the desired level of temperature, relative humidity and photoperiod can be maintained. Biochemical assay of plant defense enzymes was performed in Legumes Pathology Laboratory at ICRISAT.

PLANT MATERIAL AND SEEDLING CULTURE CONDITIONS

Pigeonpea cultivar ICP7119 (susceptible to PB) was used as a host plant to inoculate with Pdc. Seeds of cultivar was obtained from Legumes Pathology Unit, ICRISAT, Patancheru, India. For growing seedlings, sterilized plastic pots (12 cm dia) were filled with sterile alfisol and sand (9:1), five seeds (pre sterilized with 2% sodium hypochlorite for 2 min) were sown in each pot under greenhouse conditions. Ten days old seedlings were transferred to plant growth chambers and used for pathogen inoculation.

PREPARATION OF FUNGAL INOCULUM AND INOCULATION

A highly virulent isolate Pdc ICPDC1 was isolated on V8 juice agar medium. Five mm mycelial disc of actively growing Pdc ICPDC1culture was transferred into 250 ml conical flask containing 100 ml of V8 juice broth and incubated at 25 ± 2 °C and 12h photoperiod for 15 days. After 15 days, fungal inoculum (mycelial mat and broth) was thoroughly macerated using the blender until it becomes homogenous suspension.

In order to study the effect of range of temperatures on the PB development, series of treatments *viz*., six levels of temperatures 15, 20, 25, 30, 35 and 40 °C were arranged in six different CONVIRON growth chambers. Ten days old seedlings raised in the greenhouse were transferred into these growth chambers. Each pot containing five seedlings was inoculated with 100 ml of fungal suspension by soil drench inoculation method and the inoculated pots were watered liberally 3–4 times a day. Also, respective uninoculated control plants (soil drenched with water) were maintained with four replications in all the growth chambers.The plants were observed regularly for the development of disease up to 10 days and the per cent disease incidence was recorded using the formula, Disease incidence (%) = (Number of plants infected/ Total number of plants) x 100.

ENZYMES ASSAYS

Plant samples (shoot and leaves) were collected from individual treatments from 0, 24, 48, 72 and 96 hours after Pdcinoculation to study the activity of defense enzymes at range of temperatures. PAL (EC 4.3.1.5) activity was determined as per the protocol suggested by Dickerson *et al.*(1984).Assay of POX (EC 1.11.1.7) activity was carried out as per the procedure described by Hammerschmidt *et al*. (1982). The PPO (EC1.14.18.1) activity was determined as per the procedure given by Mayer *et al.* (1965). CAT (EC 1.11.1.6) activity was assayed spectrophotometrically as described by Chaparro-Giraldo *et al.* (2000).

STATISTICAL ANALYSIS

The data obtained from this experiment (per cent disease incidence and activity of defense enzymes) were subjected to analysis of variance (ANOVA) using the Statistical Analysis System (SAS) version 9.2. The data on per cent disease incidence was subjected to arcsine transformation before undergoing statistical analysis and treatment means were compared by Tukey's Studentized Range Test ($P \leq 0.05$) (SAS Institute, 2004).

RESULTS

EFFECT OF TEMPERATURES ON PB DEVELOPMENT

ANOVA shows that, interactions between the factors such as, temperature, incubation period and per cent disease incidence were significant at $P \leq 0.0001$.According to Tukey's Studentized Range Test, there is no significant difference between the temperatures 30, 35 and 40 °C in respect to the incubation period and per cent disease incidence. This experiment shows that, at 40, 35 and 30 °C, the disease starts to appear at 18, 26 and 32 hours after inoculation and achieved the 100% disease incidence within 48, 60 and 84 hours after inoculation, respectively. On the other hand, temperatures 25, 20 and 15 °C recorded only 85, 80 and 50% disease incidence after 10 days of inoculation with an incubation period of 63, 126 and 225 hours, respectively (Fig. 1). Control plants kept in all the growth chambers were healthy.

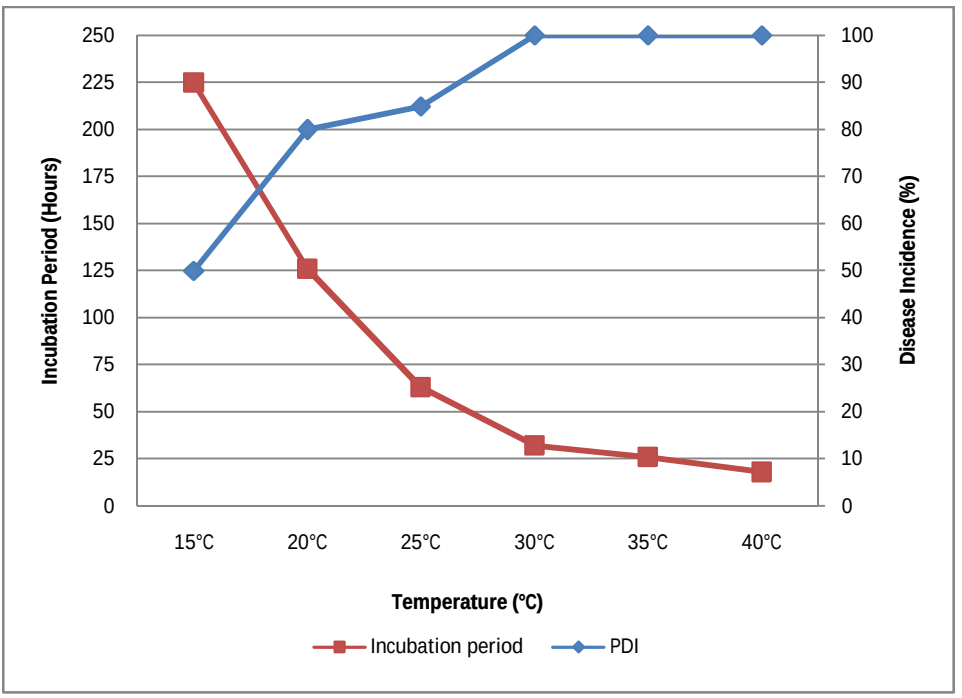


Fig. 1: Effect of Temperatures on the Interactions between Incubation Period and Incidence of PB in Pigeonpea

ACTIVITY OF DEFENSE RELATED ENZYMES

Phenylalanine Ammonia Lyase (PAL)

In general, the PAL activity was increased significantly with increasing time after Pdc infection compared to control plants irrespective of temperature. Aninduction in the PAL activity was detected and it reached the maximum level at 24 h after Pdc inoculation at 35 and 40 °C and after that a decline was noticed. However, induction was gradual and PAL activity reached maximum at 72 and 48 h after Pdc inoculation at 25 and 30 °C, respectively and the PAL activities in the controls of these temperatures were nearly the same. And, there is no much difference in the activity of PAL in the inoculated plants maintained at 15 and 20 °C up to 72 HAI and a slight induction was observed from 96 h. Interestingly it was observed that, the constitutive level of PAL activity in uninoculated pigeonpea seedlings was just starts to increase from 96 h at 15 and 20 °C, and it was starts to decrease from 96 h at 35 and 40 °C. ANOVA shows that, interactions between the factors like, temperatures, hours and activity of defense enzymes in inoculated as well as control plants were significant at $P< 0.0001$ (Fig. 2A).

Peroxidase (POX)

Induction of POX activity was differed with respect to range of temperature in the Pdc inoculated pigeonpea plants ($P< 0.0001$). The activity was found to increase upto 48 and 72 h and thereafter a decline was noticed in 25 and 30 °C, respectively. However, the activity of enzyme in plants maintained at 35 and 40 °C increased suddenly at 24 h after Pdc inoculation and then a slight reduction was noticed. Unlike other enzymes, the activity of POX enzyme in Pdc inoculated plants shows a gradual increase at 15 and 20 °C. However, POX activity was relatively constantin controls compared to inoculated plants at 25 and 30 °C. But, controls maintained at 35 and 40 °C show gradual decrease in enzyme activity from72 h ($P< 0.0001$) (Fig. 2B).

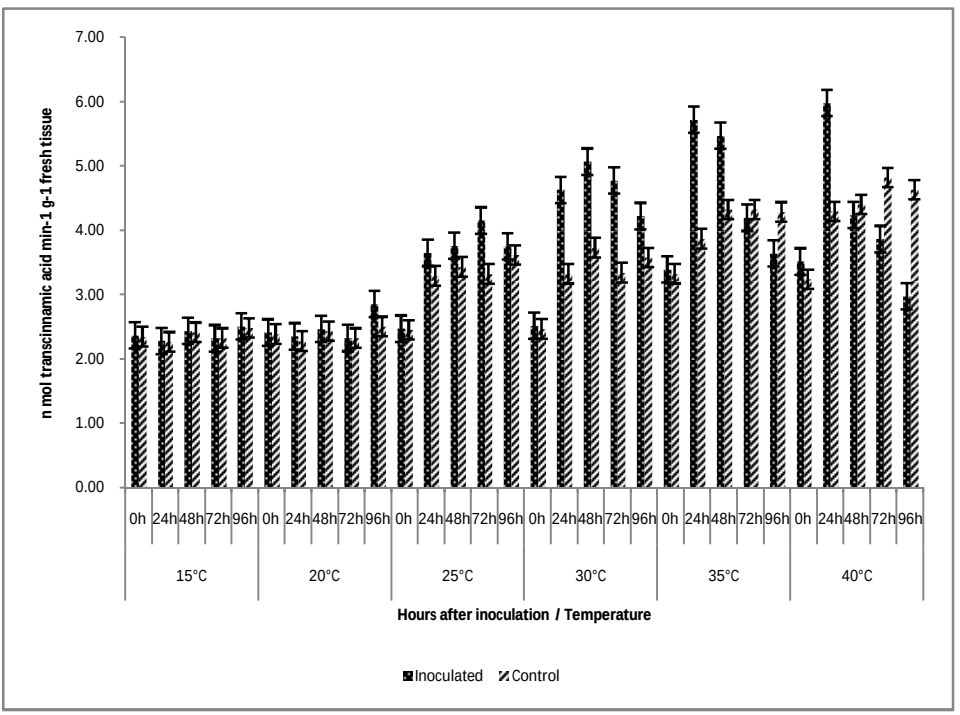
Polyphenol Oxidase (PPO)

The PPO activity reached its maximum level at 72 and 48 hours after Pdc inoculation at 25 and 30 °C, respectively and thereafter the activities starts to decrease gradually. Whereas the PPO activities reached its maximum level within 24h at both in 35 and 40 °C and thereafter the activities starts to decrease drastically ($P< 0.0001$). On the other hand, PPO activities were relatively constant in Pdc inoculated plants as well as controls at 15 and 20 °C and the controls at 25 and 30 °C showed slight increase in the enzyme activities upto 96 h (Fig. 2 C). Whereas the enzymatic activity of controls at 35 and 40 °C were stable upto 72h, after that a decline was noticed from 96h ($P< 0.0001$).

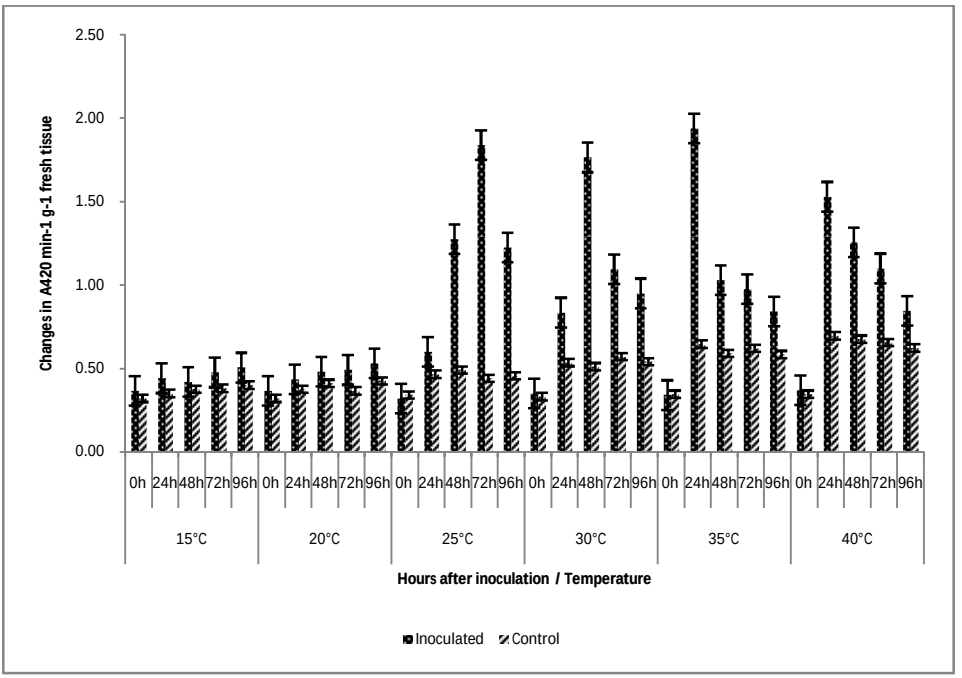
Catalase (CAT)

The activities of CAT were markedly increased in both control and inoculated plants from 25–40 °C, but the activity was significantly higher in inoculated plants compared to controls (Fig. 2 D). The pattern of changes in CAT activity was similar to that observed in PAL, showing an increase at 25 and 30 °C from 24 HAI and reached the maximum activity at 72 and 48 h, respectively and afterwards a gradual decline was noticed. Whereas the enzymatic activity reached the maximum level at 24 h after Pdc inoculation at 35 and 40 °C and then starts to decrease ($P < 0.0001$). The CAT activities in Pdc inoculated as well as control plants at 15 and 20°C were virtually similar. However, the CAT activities in the controls maintained at 25 and 30 °C were starts to increase from 72 h. On the other hand, the CAT activities in the controls of 35 and 40 °C displayed a similar trend upto 72 h and then it starts to decrease from 96 h ($P < 0.0001$).

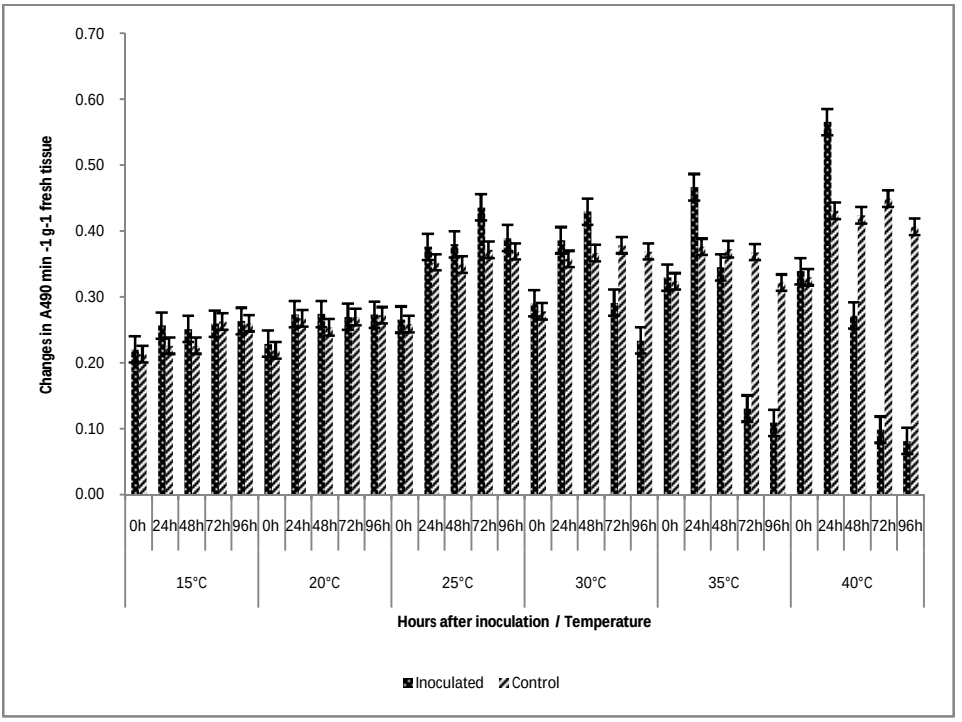
Figure 2. Induction of phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, and catalase activities in pigeonpea plants inoculated or uninoculated with Pdc. (A), (B), (C) and (D) total phenylalanine ammonia-lyase, peroxidase, polyphenol oxidase, and catalase activities in pigeonpea plants after exposed to different range of temperatures. The inoculation was done on 10 days old seedlings and the samples were collected from both un-inoculated as well as inoculated plants after specified times.



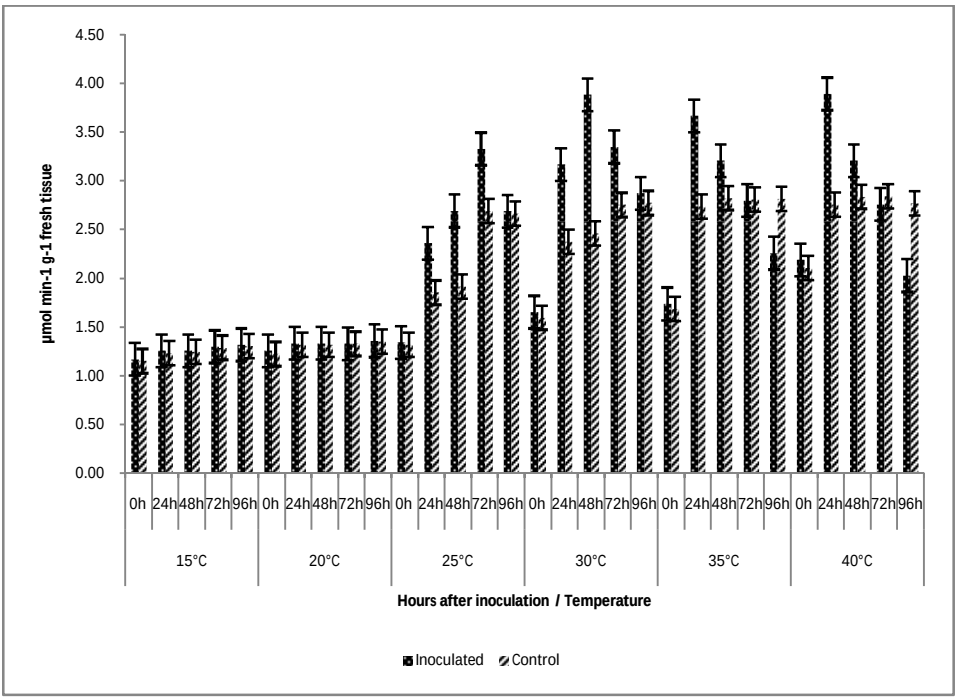
(a)


Changes in A420 min-1 g-1 fresh tissue
2.50
2.00
1.50
1.00
0.50
0.00
0h 24h 48h 72h 96h
15°C
20°C
25°C
30°C
35°C
40°C
Hours after inoculation / Temperature
Inoculated
Control

(b)


Changes in A490 min -1 g-1 fresh tissue
0.70
0.60
0.50
0.40
0.30
0.20
0.10
0.00
0h 24h 48h 72h 96h
15°C
20°C
25°C
30°C
35°C
40°C
Hours after inoculation / Temperature
Inoculated
Control

(C)



(d)

DISCUSSION

The relationship of temperature to *Phytophthora* diseases has been studied in a wide variety of crops (Tooley *et al*., 2009; Maziero *et al*., 2009; Englander *et al*., 2006; Becktell *et al*., 2005; Timmer *et al*., 2000). In this present study, the incidence of PB was noticed over the entire range of temperatures tested (15-40 °C), although amounts of disease were minor at the lower temperature (15 °C)with extended incubation period of 225h, indicating a latent infection and on the other hand, the disease incidence were 100 % at higher temperatures, 35 and 40 °C with shorter incubation period of 26 and 18h, respectively. Our result suggests that development of PB was positively correlated with increasing temperature. These results indicate that Pdc is capable of infecting pigeonpea over a wide range of temperatures. This result was supported by Granke and Hausbek (2010) who reported that *P. capsici* is capable of infecting cucumbers over a wide range of temperatures (10–35 °C). As reported by Sutherst *et al*., (2011), elevated temperatures may result in population increases due to shorter life cycles of pathogen and faster generation times, which inherently supports the current study.In contrast, Tooley *et al*., (2009), reported that disease incidence was recorded over the entire range of temperatures (10–31 °C) tested against the rhododendron plants inoculated with *P. ramorum*, but the amounts of disease were minor at the temperature extremes.

This study found that induction of four defense enzymes *viz*., PAL, POX, PPO and CAT was progressively declined after a steady increase in pathogen inoculated plants than controls with respect to range of temperatures (25–40 °C). This result is in accordance with Sutherst *et al*., (2011) who highlighted that elevated temperatures can suppress defense responses in plant hosts resulting in increased severity of disease. Similarly, Wang *et al*., (2009) found that the defense and hypersensitive responses induced by *R* genes against a bacteria (*Pseudomonas syringae*pv. *tomato*) and two viruses (*Potato virus X* and*Tobacco mosaic virus*) in *A. thaliana* and *Nicotianabenthamiana* were reduced by an increase of temperature and reported that temperature sensitivity is likely a general phenomenon in plant disease resistance against biotrophic and hemibiotrophic pathogens. We show that activities or responses of four defense enzymes to Pdc attack were inhibited by elevated temperatures and cause subsequent development in disease progress. This report is supported by a range of case studies combining biotic and abiotic stresses, stated that long-term abiotic stress can weaken plant defences and cause enhanced pathogen susceptibility (Amtmann *et al*., 2008; Goel *et al*., 2008; Wang *et al*., 2009; Mittler and Blumwald, 2010).

Overall the enzymatic activity was found to increase within 24 h in controls with respect to range of temperatures (25–40 °C), but it is started to decrease slightly from72–96 h at higher temperatures (35 and 40 °C). Hence it is assumed that scavengers or defense enzymes triggered in pigeonpea plants are being suppressed concomitantly by the invasion of Pdc as well as stress created by elevated temperatures might leads to the production of higher amount of ROS may perhaps the reason for the death of pigeonpea plants. Though, the defensive capacities of all the four enzymes were suppressed by Pdc in all the temperatures, but it was very rapid at 40 °C. Results from the present investigation show that accumulation of defense enzymes (PAL, POX, PPO and CAT) in infectedpigeonpea plants were inhibited after a steady increase and displayed a temperature mediated susceptibility to Pdc. This relationship may simply suggest that plants have developed strategies to avoid simultaneously producing proteins that are involved in abiotic stress and disease resistance responses (Anderson *et al*., 2004).

In conclusion, the relationship of temperature to Pdc has been speculated to be an important component of PB management in pigeonpea. Because, the fungus Pdc has become more aggressive when exposed to higher temperatures and at the same time, we have seen the vulnerability of pigeonpea plants to Pdc attack under higher temperatures (35–40 °C). Pdc continues to be a destructive pathogen on pigeonpea.An understanding of the effect of temperature on Pdc infection is necessary to develop management strategies including, forecast models. These results suggest that Pdc infection is influenced by increasing temperature. However, it is not known how temperature modulates or suppresses the disease resistance in pigeonpea. Does temperature affect recognition of the pathogen or does it affect the activities of defense signaling

components or what makes the pathogen become more aggressive at higher temperature? Hence, it needs further in-depth molecular study to understand the mechanisms involved during the interactions between the pigeonpea, Pdc and temperature factor.

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