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COMPARATIVE ANALYSIS OF ENZYME ACTIVITIES OF NORMAL AND *Rhizoctonia bataticola* INFECTED *Cicer arietinum* L. PLANTS AT DIFFERENT GROWTH STAGES

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Key words : *DRR, PR Proteins, Chitinases, Systemic acquired resistance.*

Chickpea (*Cicer arietinum* L.), an important cash crop, was first cultivated at least 9500 years ago in the Fertile Crescent, from Turkey to Iran, at the beginning of agriculture. Dry root rot (DRR) of chickpea caused by necrotrophic fungus *Rhizoctonia bataticola* (Taub.) Butler [Pycnidial stage: *Macrophomina phaseolina* (Tassi) Goid] is emerging as a serious threat to the chickpea production worldwide. Higher temperature and soil moisture depletion during crop growth period particularly at post-harvesting stage is predisposing chickpea to DRR. The disease is reported to be more severe when the crop is exposed to moisture stress conditions. This investigation was planned to determine the enzyme activities of normal and *Rhizoctonia bataticola* infected *Cicer arietinum* L. plants at different time intervals. Enzymes play an important role against fungal invasion and could perform defence related functions. Higher plants protect themselves against fungal infection or other biotic and abiotic factors by physical strengthening of the cell wall through lignification, suberization, and producing various pathogenesis-related (PR) proteins such as chitinases, β-1, 3-glucanases, thaumatin like proteins, etc. Plant pathogenesis-related proteins are implicated in plant defense responses against pathogen infection. Production of PR proteins in the remote uninfected parts of plants can lead to the occurrence of systemic acquired resistance, protecting the affected plants from further infection.

INTRODUCTION

Chickpea (*Cicer arietinum*) is the world's third most important pulse crop after bean (*Phaseolus vulgaris*) and peas (*Pisum sativum*), with India accounting for approximately 75% of the world's chickpea production (FAO, 1993). Chickpea (*Cicer arietinum* L.), also called garbanzo bean or Bengal gram, is an Old World pulse and one of the seven Neolithic founder crops in the Fertile Crescent of the Near East (Lev-Yadun *et.al.,* 2000). Chickpea (*Cicer arietinum* L.) is one of the most important grain legumes in India, grown in an area of 13.2 million ha with an annual production of 11.60 million tons of grain (FAOSTAT, 2013). South Asia is the largest producer of chickpea (76%) and India is the largest chickpea growing country with an annual production of 7.70 million tons from 8.32 million hectares (FAOSTAT, 2012). The other major chickpea producing countries include Pakistan, Turkey, Australia, Myanmar, Ethiopia, Iran, Mexico, Canada and USA. In the semi-arid tropics, chickpea is an important component of the diets of those individuals who cannot afford animal proteins or those who are vegetarian by choice. Chickpea is a good source of carbohydrates and proteins, together constituting about 80% of the total dry seed mass in comparison to other pulses. Chickpea is cholesterol free and is a good source of dietary fibre, vitamins and minerals (Wood & Grusak, 2007). Globally, chickpea is mostly consumed as a seed food in several different forms and preparations are determined by ethnic and regional factors. The average production of chick pea is 25-30 quintals per hectare which is low in spite of high yielding varieties and new agronomic practices. Low yields are attributed to different factors, among which pathogenic micro-organisms and insect attacks are considered the most serious. The reasons of low yield are widely variable; the main factor being the incidence of diseases. The production of chickpea is largely constrained by dry root rot (DRR), a soil borne disease caused by *Rhizoctonia bataticola*. However, recently wilt resistant chickpea cultivars were found succumbing to dry root rot (DRR) in major chickpea growing regions (Pande & Sharma *et al.* 2010).

The DRR is caused by a necrotrophic fungus *Rhizoctonia bataticola* (Taub.) Butler [Pycnidial stage: *Macrophomina phaseolina* (Tassi) Goid] and is an important component of the disease complex that causes root rots and seedling blight in many grain legumes when they are weakened by other stress factors (Hawang *et al.* 2003). In the absence of the host crop, it survives in soil as a competitive saprophyte on available dead organic matter. A critical analysis of the weather data (2000-2010) of the major chick- pea growing areas in India indicated higher incidence of DRR in years when temperature exceeds 33°C (Pande *et al.* 2010). The DRR was not of much significance in chickpea earlier; however, it has become a major threat to chickpea production in recent years due to altered weather conditions, particularly on account of longer drought spells. Higher temperature and soil moisture depletion during crop growth period particularly at post-harvesting stage are predisposing chickpea to DRR (Sharma and Pande, 2013).

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MATERIALS & METHODS

COLLECTION OF DISEASED MATERIAL

Naturally infected Chick pea plants, showing characteristic symptoms of Dry Root Rot were collected from the field of Pulse Section, Bihar Agriculture College, Sabour, Bhagalpur, India which were surveyed. Such *Rhizoctonia bataticola* affected plants were brought to the laboratory. Dry Root Rot disease caused by *Rhizoctonia bataticola* was observed on variety PG-256 in Sabour, Bhagalpur during March-April 2013.

Fungal Isolate

A pathogenic isolate of *Rhizoctonia bataticola*, isolated from naturally infected chickpea plant at Bihar Agriculture College, Sabour, Bhagalpur, India was used throughout the experiments. Isolate was purified using mono-sclerotia and maintained on PDA slants at 5°C in refrigerator.

Seed germination studies

Seeds of chickpea cultivars were surface sterilized, washed and soaked in sterile distilled water and allowed to germinate. Germinated seeds at different time intervals were collected separately, frozen in liquid nitrogen, powdered by grinding with acetone, and finally defatted with hexane. Seeds (2 g) washed thoroughly after surface sterilization, were also soaked overnight in 12 ml of sterile distilled water and the water containing proteins leached out of the seeds was decanted. The proteins in the extract were precipitated with ammonium sulphate at 0.9 saturation by adding solid ammonium sulphate (60 g per 100 ml extract). The precipitated proteins were pelleted by centrifugation after being stored at 4°C overnight and resuspended in a minimum volume of 0.1 M phosphate buffer, pH 6.9, containing 0.05 M NaCl. The suspension was dialysed against the same buffer with three changes at 6-h intervals and the supernatant obtained after centrifugation was used to estimate enzyme activities. Protein concentration in samples was estimated using bovine serum albumin as standard (Bradford, 1976).

Plant growth and inoculation with pathogen

For determining the induction of antifungal proteins in response to pathogen infection, the surface sterilized, presoaked seeds of PG-256 cultivar were grown in pots containing soil.

The root, shoot and cotyledon tissues were collected separately at different growth phases after germination (DAG) and were frozen immediately in liquid nitrogen to store at 20°C. The tissues were treated extensively with acetone for depigmentation and dehydration, followed by defatting with hexane.

ENZYME ACTIVITIES

α -Amylase Activity Assay

α-Amylase activity was determined according to the method of Jones & Varner (1967). Seeds were extracted in 0.2 M citrate buffer (pH 5.5), centrifuged at 10,000 g and the supernatant was used for enzyme assay. Then 0.2 ml of the enzyme extract was diluted to make the volume 1.0 ml with distilled water. The reaction was started by the addition of 1.0 ml of starch substrate for one hour. The starch substrate was prepared by the addition of 150 mg potato starch in 100 ml of solution containing 600 mg KH_2PO_4 and 200 µmol $CaCl_2$. The mixture was boiled for 1 minute, centrifuged for 10 minutes at 3,000 g and clear supernatant was used as the substrate. The reaction was stopped by the addition of 1 ml of iodine reagent (6 g of KI and 600 mg of iodine were dissolved in 100 ml of water, before use 1.0 ml of the stock solution was added to 0.05 N HCl and made the volume to 100 ml). To this reaction mixture, 5.0 ml of distilled water was added, mixed and measured the absorption at 620 nm. The α-amylase activity was calculated as the amount of starch hydrolyzed per minute per mg of protein. Protein content was determined according to the method of Bradford (1976) with BSA as a standard.

β -Amylase Activity Assay

β-amylase activity was measured using Bernfeld's method (1955). Reaction mixture was prepared by taking 0.5 ml of suitably diluted enzyme and 1% starch prepared in 50 mM sodium acetate buffer, pH 5.0. This was incubated at 30°C for 3 min, reaction was stopped by addition of 1 ml of 3, 5-dinitrosalicylic acid. Test tubes were then placed in boiling water bath for 5 min and were allowed to cool down to room temperature, followed by addition of 10 ml of distilled water. Absorbance was recorded at 540 nm. One unit of β-amylase is defined as the amount required for release of 1 µM of β-maltose per min at 30°C and pH 5.0 under the specified condition. Amount of protein present in sample was determined by Folin's method using crystalline BSA as standard protein.

Protease Activity Assay

Preparation of crude enzyme extract seeds (2g) was ground in a mortar with cold 0.1M phosphate buffer of pH 7.0 and finally crushed into paste using a homogenizer. The temperature was maintained at 4°C by putting ice in the outer chamber of the homogenizer. The suspension was then filtered through few layers of cheese cloth in the cold room. The filtrate was collected and clarified further by centrifugation in a refrigerated centrifuge at 10,000 rpm for 15 min at 4°C. Protease activity was assayed by the method as described (Reimerdes and Meyer, 1976) using milk protein casein as substrate. The protease activity was measured by estimating the release of tyrosine calculated from the standard curve prepared with tyrosine. One unit of protease activity was defined as the amount required for liberating 1 mg of tyrosine in 30 min at 45°C.

Chitinase Activity Assay

Chitinase activity was determined as described by Chen *et al.* (1982) and Tsukomoto *et al.* (1984). The reaction mixture contained 1.0 ml of colloidal chitin solution (7 mg), 1.0 ml of sodium acetate buffer (50 mM, pH 5.2) and 1.0 ml of suitably diluted enzyme. After incubation at 50°C for 1 h the released reducing sugar was measured as N-acetylglucosamine (NAG) equivalents by the Somogyi-Nelson method (Somogyi, 1952). The assay mixture contained 1.0 ml of suitably diluted enzyme and 1.0 ml of 1% laminarin solution in sodium acetate buffer (50 mM, pH 5.2). The mixture was incubated at 40°C for 30 min and the released reducing sugar was measured as glucose equivalents (Somogyi, 1952). One unit (U) of chitinase was defined as the amount of enzyme that produced one mg of NAG per hour under the given assay conditions.

RESULTS & DISCUSSION

Enzyme Activities of normal and infected chickpea at different growth phases.

α-Amylase activities (Maltose Unit gm^{-1} dry matter) of normal and infected chickpea at different growth phases.

All mean scores bearing different superscripts in rows are significantly different (P< 0.05).

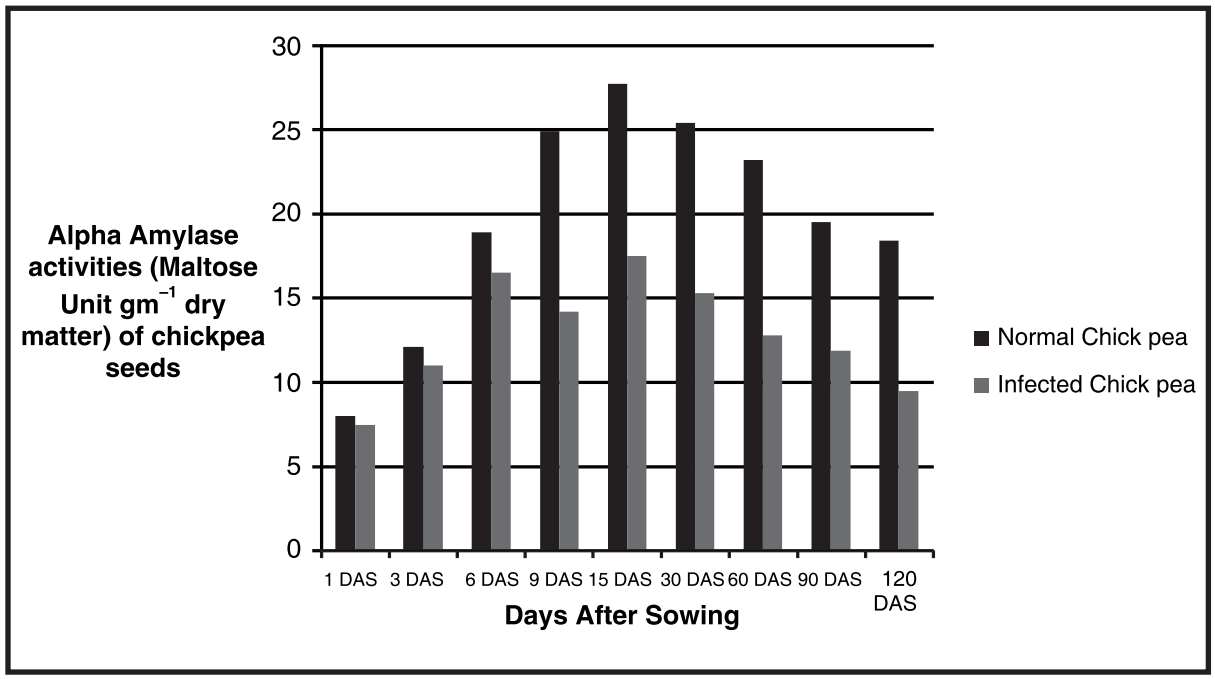

Fig 1: Graph represents the α-amylase activity of normal chickpea in comparison to infected chickpea at different stages of growth phases.

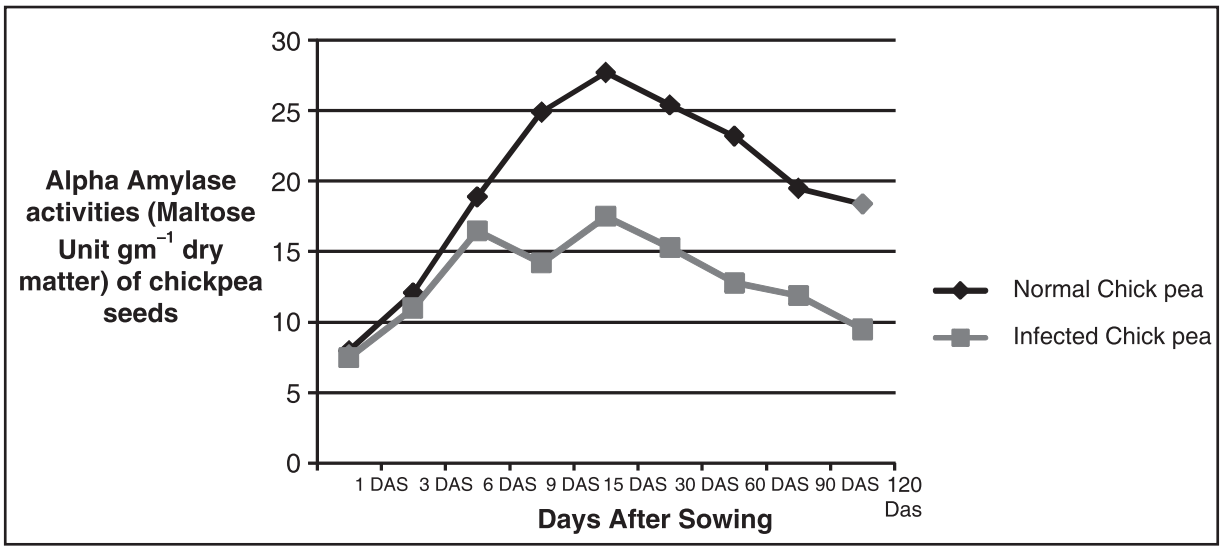

Fig 2: Comparison of normal and *Rhizoctonia bataticola* infected chickpea cultivars in terms of α-amylase activity at different stages of growth phases.

In Fig.1 & 2, the normal seeds of the chickpea exhibited α-amylase activity that ranged from 6.5 to 27.7 maltose units/ g dry matter. The highest (P 0.05) alpha amylase activity of 27.7 maltose units/g dry matter was with normal seeds of chickpea cultivar. Normal chickpea had significantly the least enzymatic activities (6.5 units/g dry matter) (Fig 1). In this graph the α-amylase activities of normal chick pea is 8.0 Maltose Unit g^{-1} dry matter increases from 1st DAS and had attained a maximum at 15 DAS, i.e., 27.7 Maltose Unit g^{-1} dry matter and after that α-amylase activity had begun to decline and the value reaches to 18 Maltose Unit g^{-1} dry matter at 120 DAS. In infected chickpea with *Rhizoctonia bataticola* the α-amylase activity is 7.5 Maltose Unit g^{-1}dry matter which increases from 1 DAS and had attained a maximum at 15 DAS, i.e., is 17.5 Maltose Unit^{-1} dry matter and after that α-amylase activity had begun to reduce significantly.

In normal chick pea by the α-amylase activity, carbohydrate deposition breaks down and releases glucose which enters into respiratory channel through Glycolysis, Krebs cycle, electron transport chain and oxidative phosphorylation for the production of ATPs and formation of chemical compounds that undergo for the operation of biochemical reaction but in infected seeds with *Rhizoctonia bataticola*, the carbohydrate decomposition is consumed by the hyphae and sufficient amount of ATPs are not produced due to blockage of respiratory channel that results into dry root rot of the plant. α-Amylase has an active role in the hydrolysis of the starch during seed germination. It may also be responsible for the maintenance of requisite water potential, by providing soluble sugars during the seed germination phase. Strong starch degrading activity is present in the cell wall of plants.During germination of seeds, α-amylase and protease, that are developed, degrade starch granules and reserve proteins, respectively; thereby reducing the dietary bulk and improving the digestibility of starch and protein [Reihaneh and Mehdi (2011); Negi *et al.,*(2001); Lasekan, (1996); Muntz (1996); Kataria, *et al.,* (1992)]. Germination was shown to increase monosaccharide and decrease disaccharide contents of legumes due to α-amylase [Akinlosottu and Akinyele (1991)]. Monosaccharides can be used as a good energy source and will also increase the palatability of seeds.

β- Amylase activities (Maltose Unit gm^{-1} dry matter) of normal and infected chickpea at different growth phases.

All mean scores bearing different superscripts in rows are significantly different (P< 0.05).

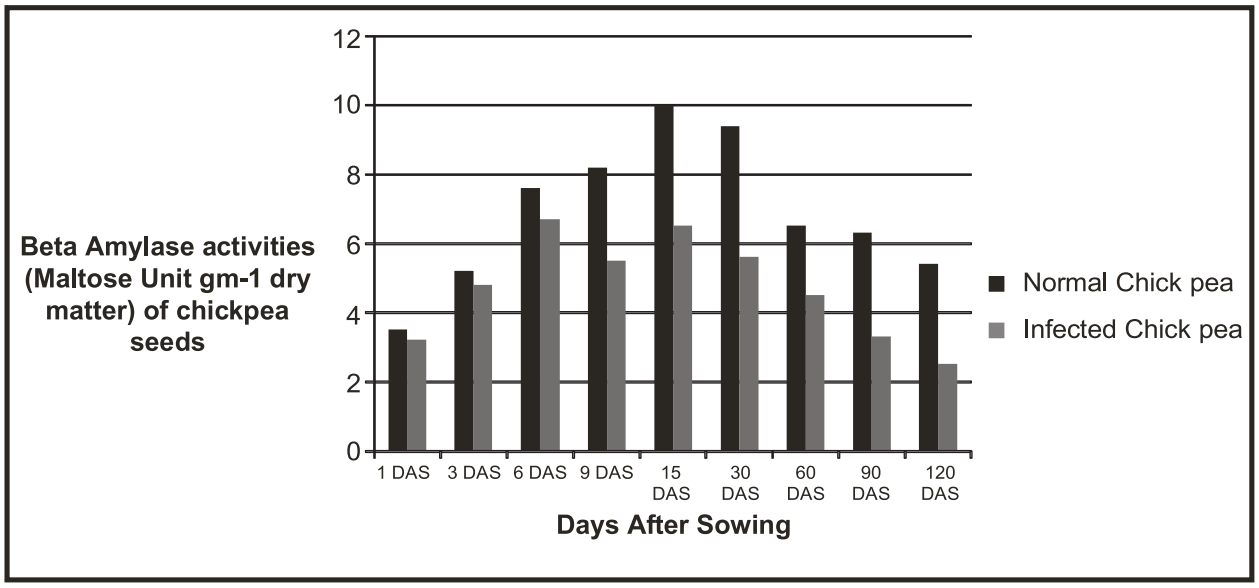

Fig 3: Graph represents the α-amylase activity of normal chickpea in comparison to infected chickpea at different stages of growth phases.

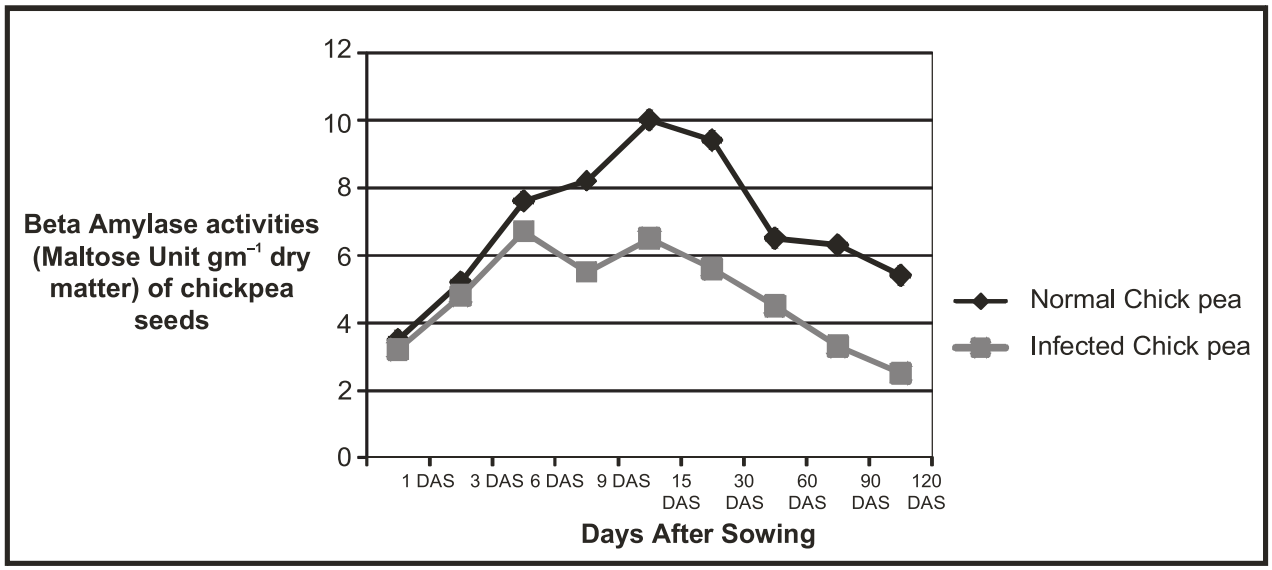

Fig 4: Comparison of normal and *Rhizoctonia bataticola* infected chickpea cultivars in terms of β-amylase activity at different stages of growth phases.

It was observed that the pattern of changes in β- amylase activities in normal and *Rhizoctonia bataticola* infected chickpea cultivars of PUSA-256 were as shown in Fig.3 & 4. β- amylase activity of normal chickpea cultivars varied from 2.7 to 10 Unit gm^{-1} seed powder; whereas it was in the range 2.4 to 6.5 Unit gm^{-1} seed powder in *Rhizoctonia bataticola* infected chickpea cultivars. It was found that the β - amylase activity of normal chickpea is higher than the infected cultivars of chickpea. In normal chickpea cultivars, β- amylase activity increases gradually after seed soaking and reaches maximum activity at 15 DAS (10 U gm^{-1} seed powder) and then the activity reduces to 5.4 U gm^{-1} seed powder at 120 DAS; whereas in infected chickpea cultivars the β- amylase activities increased gradually after soaking (3.2 U gm^{-1} seed powder) at 1 DAS to 6.5 U gm^{-1} seed powder at 15 DAS then returned to 2.5 U gm-1 seed powder at 120 DAS.

Maximum activity was found in both normal and infected cultivars at 15 DAS. It was found that the β- amylase activity showed an increasing trend upto 15 DAS, thereafter decline was observed (Fig.3 & 4). β- Amylase was found to be present in the ungerminated seed, which did not contain starch. Similar to other plant β- amylases, Chickpea β- amylase also showed inability towards degradation of the native starch granule. Therefore, presence of not high but significant amount of α- amylase may be required to initiate the reaction, followed by hydrolysis by β- amylase. At this stage of germination, starch acts as carbohydrate reserve and thus its hydrolysis is needed for germination and growth of seedling. The high amount of β -amylase present as compared to α- amylase and its presence at the periphery of amyloplasts defines the key role of β- amylase in hydrolysis of starch. A decline in the content of starch and β-amylase was observed at this stage.

Protease activities (Unit gm^{-1} seed powder) of normal and infected chickpea at different growth phases.

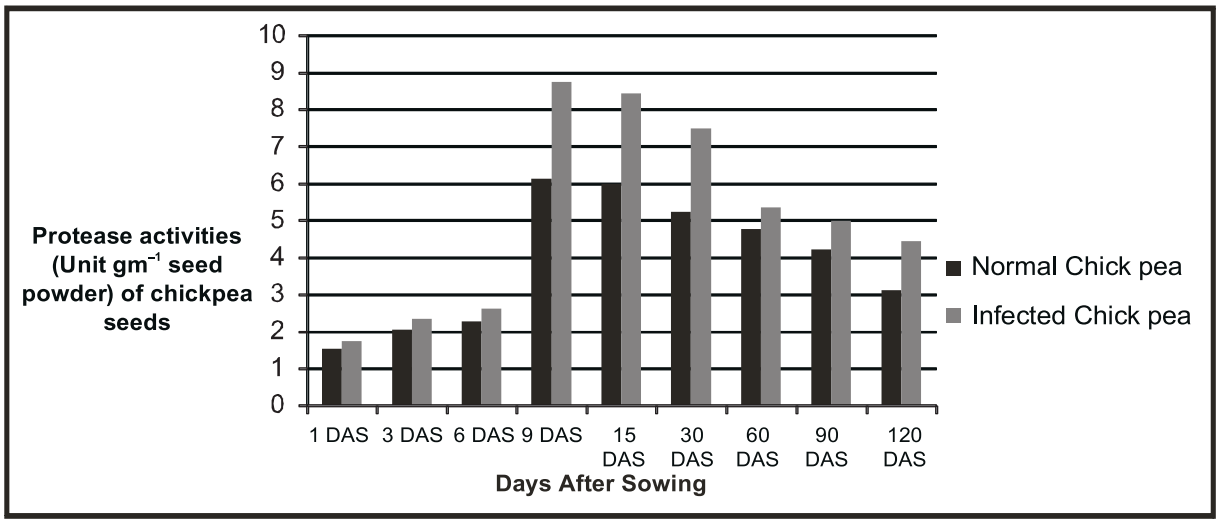

Fig 5: Graph represents the protease activity of normal chickpea in comparison to infected chickpea at different stages of growth phases.

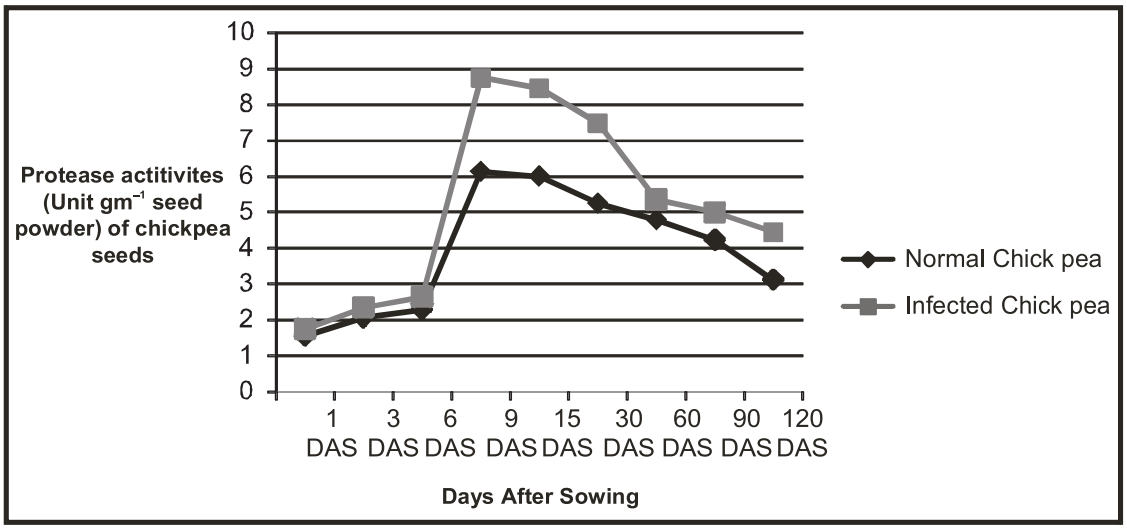

Fig 6: Comparison of normal and *Rhizoctonia bataticola* infected chickpea cultivars in terms of protease activity at different stages of growth phases.

The observation that the protease activities in infected chickpea cultivar was higher than the normal cultivar indicated clearly that it was resulting in increase in the antifungal activity, and having a direct role in hydrolysing toxic proteins of fungal origin. The pattern of changes in protease activities in normal and *Rhizoctonia bataticola* infected chickpea cultivars of PUSA-256 were as shown in Fig.5 & 6. Protease activity of normal chickpea cultivars varied from 1.45 to 6.13 Unit gm^{-1} seed powder; whereas it was in the range 1.58 to 8.75 Unit gm^{-1} seed powder in *Rhizoctonia bataticola* infected chickpea cultivars. It was found that the protease activity was higher in infected chickpea than in the normal cultivars of chickpea. In normal chickpea cultivars protease activity increases gradually after seed soaking and reaches maximum activity at 9 DAS (6.13 U gm^{-1} seed powder) and then the activity reduces to 3.12 U gm^{-1} seed powder at 120 DAS; whereas in infected chickpea cultivars the protease activities increased gradually after soaking (1.75 U gm^{-1} seed powder) at 1 DAS to 8.75 U gm^{-1} seed powder at 9 DAS then returned to 4.45 U gm^{-1} seed powder at 120 DAS. Maximum activity was found at 9 DAS in infected cultivars. Proteases may have a direct role in hydrolysing proteins secreted by invading pathogens, thereby preventing pathogenesis.

Constitutive expression of proteases required for processing and scavenging of PRPs was described by Rodrigo *et al*. (1989, 1991). It was demonstrated that the protease was specifically produced upon infection by *Rhizoctonia bataticola*. Proteases are also involved in the complex process of signal transduction where they may participate in the recognition and processing of systemin, the peptide hormone leading to activation of a cascade of cellular events affecting the defence reaction (Schallar and Ryan, 1996). Genes encoding leucine aminopeptidase or aspartic protease or subtilisin-like proteases are reported to be activated during the defence response of tomato plants (Schallar and Ryan, 1996; Tornero *et al.* 1996, 1997). Because of their possible importance in defence against pathogens, further characterization of proteases specifically induced in roots of resistant chickpea cultivars is in progress. Knowledge of the role of proteases in activating PRPs or in recognizing the pathogen and in signalling will be useful for planning strategies for developing resistance of chickpeas to pathogen infection.

Chitinase activities (Unit gm^{-1} seed powder) of normal and infected chickpea at different growth phases.

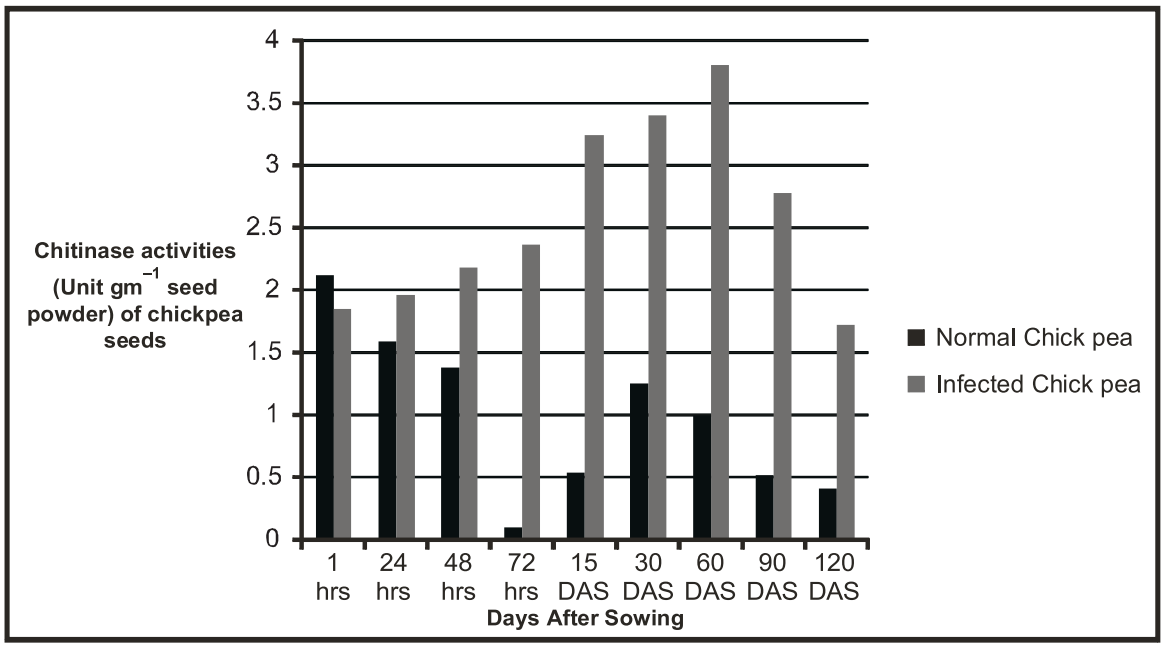

Fig 7: Graph represents the chitinase activity of normal chickpea in comparison to infected chickpea at different stages of growth phases.

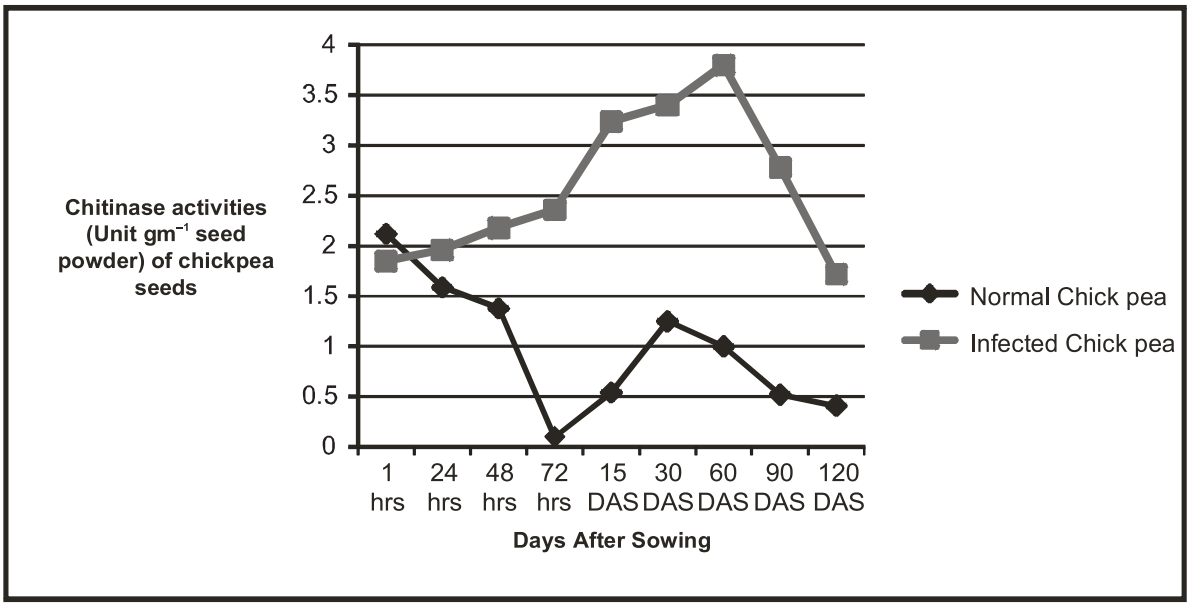

Fig 8: Comparison of normal and *Rhizoctonia bataticola* infected chickpea cultivars in terms of chitinase activity at different stages of growth phases.

The pattern of changes in chitinase activities in normal and *Rhizoctonia bataticola* infected chickpea cultivars of PUSA-256 were as shown in fig.7 & 8. Chitinase activity of normal chickpea cultivars varied from 0.10 to 2.12 Unit gm^{-1} seed powder; whereas it was in the range 1.72 to 3.80 Unit gm^{-1} seed powder in *Rhizoctonia bataticola* infected chickpea cultivars. It was found that the chitinase activity was higher in Infected chickpea than in the normal cultivars of chickpea. In normal chickpea cultivars chitinase activities declined gradually after seed soaking and then the activity increases from 15 DAS (0.54 U gm^{-1} seed powder) to 30 DAS (1.25 U gm^{-1} seed powder) and then again the activity reduces to 0.41 U gm^{-1} seed powder at 120 days after sowing. Proteins leached from seed of normal chickpea cultivar showed the presence of chitinase activity; whereas in infected chickpea cultivars the chitinase activities increased gradually after soaking (1.85 U gm^{-1} seed powder) at 1 DAS to 3.80 U gm-1 seed powder at 60 DAS then returned to 1.72 U gm^{-1} seed powder at 120 DAS. Maximum activity was found at 60 DAS in challenged cultivars. In this case mycelium of *Rhizoctonia bataticola* are ramifying and chitinase activity becomes very high that act on chitin wall of fungus. It has been observed that in infected chick pea as DAS increases the chitinase activity also increases.

Interaction between a plant and a pathogen leads to the induction of numerous host-specific biochemical responses, some of which are critical for the plant to withstand the attack of the pathogen (Dixon and Lamb, 1990; Dangl and Holub, 1997), such as the production of chitinases and β-1,3-glucanases (Bol *et al*. 1990; Bowles, 1990). Many phytopathogenic fungi contain chitin and glucan as components of their cell wall polymers. Depolymerization of cell walls by the combined activity of chitinase and glucanases was reported to kill fungi in vitro (Schlumbaum *et al*. 1986; Mauch *et al*. 1988). Cell wall fragments were shown to induce defence reaction in plants by switching on genes responsible for the synthesis of pathogen-related proteins (PRPs) [Ryan and Farmer, 1991].

Levels of chitinases and β-1,3-glucanase are also known to be reduced in a resistant cultivar as the infection is confined. In susceptible plants, on the other hand, high levels are maintained as the pathogen remains active in the plant tissue (Beckman & Roberts, 1995). The observed decrease in chitinase activity in the infected cultivar may be associated with the reduction in the pathogen invasion as a result of the defence reaction.

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