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Fitoplazma ile enfekte olmuş farklı Desi ve Kabuli tipi nohut genotiplerinin yapraklarındaki biyokimyasal değişiklikler

Research Article

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Abstract

Objective: Chickpea phyllody caused by phytoplasma occurs worldwide. However, the alterations in the host physiology and its associated biochemical components induced by the infection with phytoplasma in chickpea plant remain unknown.

Methods: In present study, the changes in phenolic compounds, protein contents, phenylalanine ammonia-lyase (PAL), peroxidase (POX), catalase (CAT), polyphenol oxidase (PPO) and chlorophyll contents were analyzed in phytoplasma-symptomatic and non-symptomatic plants of two Kabuli and three Desi type chickpea genotypes.

Results: Total phenols were statistically at par in symptomatic plants of both Kabuli and one Desi (Bittle-98) genotype but significantly increased in genotype Aug-424 and decreased in C-727. Protein contents were significantly decreased in symptomatic plants of all genotypes except CM-2008. PAL activity was significantly increased in all Kabuli but decreased in all the Desi genotypes. POX activity was significantly increased in Noor-2009 and Aug-424 but decreased in CM-2008 and Bittle-98. PPO activity was increased in two genotypes but decreased in others. CAT activity and chlorophyll contents were decreased in all genotypes.

Conclusion: The present finding indicates that phytoplasma causes non-specific, general stress response by interfering with host metabolism and photosynthesis. The study also provided significant insights for better understanding the mechanisms of chickpea plant response to phytoplasma.

Keywords: Cicer arietinum; Phyllody; Phytoplasma; Biochemical changes; Pakistan.

Özet

Amaç: Fitoplazma'nın neden olduğu nohut bitkisinde oluşan phyllodi dünya genelinde görülmektedir. Ancak, nohut bitkisinde fitoplazma enfeksiyonu ile indüklenen konukçu fizyolojisinde ve buna bağlı biyokimyasal bileşenlerdeki değişiklikler bilinmemektedir.

Yöntemler: Bu çalışmada, fenolik bileşikler, protein içerikleri, Fenilalanin amonyak-liaz (PAL), peroksidaz (POX), katalaz (CAT), polifenol oksidaz (PPO) ve klorofil içeriklerindeki değişiklikler fitoplazma-semptomatik ve semp-tomatik olmayan iki Kabuli ve üç Desi türü nohut genotipinde çalışıldı.

Bulgular: Toplam fenoller, hem Kabuli hem de bir Desi (Bittle-98) genotipinin sempomatik bitkilerinde eşit düzeyde bulunurken, Aug-424 genotipinde anlamlı olarak artmış, C-727’dede ise azalmıştır. CM-2008 dışındaki tüm genotiplerin sempomatik bitkilerinde protein içeriği önemli ölçüde azalmıştır. PAL aktivitesi tüm Kabuli genotiplerinde önemli ölçüde artmış, ancak tüm Desi

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genotiplerinde azalmıştır. Noor-2009 ve Aug-424′de POX aktivitesi önemli ölçüde artmış ancak CM-2008 ve Bittle-98′de azalmıştır. Tüm genotiplerde CAT aktivitesi ve klorofil içeriği azalmıştır.

Sonuç: Bu bulgular, fitoplazmanın konukçu metabolizmasına ve fotosenteze müdahale ederek spesifik olmayan, genel stres tepkisine neden olduğunu göstermektedir. Çalışma ayrıca, nohut bitkisinin fitoplasmaya tepki mekanizmalarını daha iyi anlamak için önemli bilgiler sağlamıştır.

Anahtar Kelimeler: Cicer arietinum; Phyllodi; Fitoplasma; Biyokimyasal değişiklik; Pakistan.

Introduction

Chickpea (Cicer arietinum) is an important, cool-season legume of versatile food use that ranks third in the world. It is a rich source of good quality protein with ability to sustain soil fertility when included in different cropping systems. It is mostly grown under rain fed conditions in arid and semi-arid areas around the world [1]. The major producers of chickpea are India, Pakistan and Turkey. In Pakistan it covers an area of 9.85 million hectares with an average annual production of 6.73 million tons. The yield of chickpea in Pakistan is already low due to a series of biotic and abiotic stresses. More than 50 diseases and 54 insect pests have been reported to date on chickpea in different parts of the world [2]. The situation is further aggravated due to the recent prevalence of 16SrII-D phytoplasma associated with chickpea phyllody disease in Pakistan [3].

The phytoplasmas are a group of plant pathogenic wall-less, phloem inhabiting bacteria in the class mollicutes that are naturally transmitted by phloem feeding insects, mostly leafhoppers [4]. Phytoplasmas can cause devastating damage to plants by loss in biomass and quality of plant products including flowers. Currently, there are no appropriate measures for directly controlling phytoplasma-caused disease. The disease could potentially be managed indirectly by spraying systemic insecticides to kill the leafhopper vector but the use of resistant germplasm is the best option.

In Pakistan phytoplasma was found to infect plants of both Kabuli and Desi type chickpea for the first time during 2005 [5]. The major symptoms observed were floral virescence, phyllody and extensive proliferation of branches. At the time of crop maturity when the healthy plants are drying, the diseased plants in the field remain green. Chickpea phyllody disease in Pakistan was reported to be caused by 16SrII-D phytoplasmas which was found to be transmitted by a leafhopper Orosius albicinctus [3].

Phytoplasmas are introduced directly into plant phloem sieve tube cells during vector feeding and translocate with phloem sap in the direction of photosynthetic “sinks”. Phytoplasmal infection severely damages the physiological and biochemical process of plants [6]. One of the main effects of phytoplasmal infection is the decrease in plant productivity caused by inhibition of photosynthesis [7]. This decline of photosynthesis can be a result of the direct effect of infection on photosynthetic electron transport and enzymatic activities [6].

The occurrence of phyllody disease of chickpea is a new entrant to disease scenario in Pakistan [3]. It has also been reported from Coimbatore in Tamil Nadu, India during 1959 [8]. This syndrome has been observed in Ethiopia, Myanmar, Australia and Oman [3]. The association of a phytoplasma was confirmed by molecular techniques using PCR and sequence information’s. However, the alterations in the host physiology and its associated biochemical components induced by the infection with this recently known 16SrII-D phytoplasma in Pakistan in chickpea plant remain unknown. In this view, the present study was performed to understand the biochemical basis of phyllody disease in chickpea (Kabuli and Desi types) using diseased and healthy plants. Based on alterations in various biochemical activities, possible mechanism of phyllody disease susceptibility was described.

Materials and methods

Plant material

Seeds of two Kabuli (Noor-2009, CM-2008) and three Desi (Bittle-98, Aug-424, C-727) type chickpea genotypes were collected from pulses breeders at Nuclear Institute for Agriculture and Biology, Faisalabad, Pakistan (NIAB) and were grown in field. Observations for phyllody disease were started 2 weeks after germination and both symptomatic and non-symptomatic plants were tagged in the naturally infected fields at early growth stage to collect samples to determine the patho-physiological changes that take place.

Total phenols

One gram leaf samples from phytoplasma infected and healthy plants of each test genotypes were collected
after pod formation on healthy plants. Leaves were cut into small pieces and then put into the smearing methanol until the green color is extracted. Leaf tissues were homogenized after decanting the methanol in the Polytone (PT1600E) and then again boiled in methanol for further 5 min and filtered through Whatman No. 1 filter paper. Residual material was washed with 80% acidified (0.1% HCL conc.) methanol. Using rotavapour (BUCHI R-114), methanol was evaporated and the aqueous layer was collected to adjust final volume as mL/g of weight with distilled water. Aqueous portion of extract was then washed with n-hexane to remove green color. The concentration of phenolic compounds in the leaves of phytoplasma infected and healthy plants was determined by using the folin-ciocalteau reagent. The 0.2 mL of above prepared leaf extract were taken in test tubes and added 4 mL of 4% Na2CO3 solution then 0.2 mL of folin-ciocalteau reagent was added after 3 min with constant shaking on vortex mixer. Absorption was measured in Double Beam Spectrophotometer (Hitachi u-2800) at 750 nm after 30 min. Chlorogenic acid was used as standard and the total phenolic concentration was calculated as mg/g fresh weight of chickpea leaves [8].

**Protein activity**

Fully emerged leaves (0.5 g) from phytoplasma infected and healthy plants of each test genotypes were ground in cold extraction buffer. Samples were centrifuged at 15,000 \( \times \) \( g \) for 10 min at 4°C and the supernatant was separated. Total soluble protein concentration was measured by dye binding assay as described by Bradford [9].

**Phenylalanine ammonia-lyase (PAL)**

PAL activity was assayed following the earlier method reported by Ngadze et al. [10]. Leaf tissue samples (2.5 g) from phytoplasma infected and healthy plants of each test genotypes were cut into small pieces of about 5 mm long and it was ground with 5 mL of a buffer containing 50 mM Tris, 15 mM 2-mercaptoethanol, and 5% polyvinyl polypyrrolidone (Sigma) and filtered through four layers of muslin cloth. The filtrate was centrifuged at 13,000 rpm for 5 min at 4°C. One milliliter of supernatant was mixed with 2 mL of 0.05 M borate buffer (pH 8.8) and 1 mL of 0.02 M L-phenylalanine. The samples were incubated at 30°C for 1 h. The reaction was stopped by adding 0.2 mL of 6 M trichloroacetic acid (TCA) and PAL activity was measured after 2 h of sectioning. One activity unit was defined as a change in absorbance of 0.01 at 290 nm h \(^{-1} \) g \(^{-1} \) protein [8].

**Peroxidase activity (POX)**

For the estimation of POX, leaves from phytoplasma infected and healthy plants of each test genotypes were homogenized in a medium composed of 50 mM potassium phosphate buffer (pH 7.0), 0.1 mM EDTA and 1 mM dithiothreitol (DTT). Activity of POX was measured using the method described by Chance and Maehly [11] with some modification. For measurement of POX, activity assay solution (3 mL) contained 50 mM phosphate buffer (pH 7.0), 20 mM guaiacol, 40 mM \( \text{H}_2\text{O}_2 \) and 0.1 mL enzyme extract. The reaction was initiated by adding the enzyme extract. Increase in absorbance of the reaction solution at 470 nm was recorded after every 20 s. One unit POX activity was defined as an absorbance change of 0.01 units min \(^{-1} \) [8].

**Catalase activity (CAT)**

Phytoplasma infected and healthy plants of each test genotypes were thoroughly mixed in a medium composed of 50 mM potassium phosphate buffer, pH 7.0 and 1 mM dithiothreitol (DTT) for the measurement of CAT activity. Assay solution (3 mL) contained 50 mM phosphate buffer (pH 7.0), 5.9 mM \( \text{H}_2\text{O}_2 \) and 0.1 mL enzyme extract. Reduction in absorbance of the reaction solution at 240 nm was recorded after every 20 s. An absorbance change of 0.01 unit’s min \(^{-1} \) was defined as one unit CAT activity. Enzyme activity was expressed as on fresh weight basis [12].

**Polyphenol oxidase activity (PPO)**

To measure PPO activity leaf tissue samples were cut into small pieces of about 5 mm long from each of the treatment and were ground in liquid nitrogen using a mortar and pestle. PPO activity was measured as described by Ngadze et al. [10]. The absorbance at 546 nm was measured for 4 min at 20 s intervals, and the values per minute were calculated and the results were presented as U \( \mu \)L \(^{-1} \) min \(^{-1} \).

**Chlorophyll contents**

The chlorophyll a, chlorophyll b and total chlorophyll contents of leaves were estimated from both phytoplasma
infected and healthy plants of each test genotypes using standard procedures [13, 14]. For these purpose leaf samples of 0.25 g were ground in 10 mL of 80% acetone, ground in the presence of sand in pestle and mortar and then filtered through muslin cloth. The absorbance of extract was measured at 663, 645, 505, 453 and 470 nm wavelengths, and above mentioned pigments were calculated according to standard procedure [15].

**Statistical analysis**

The data collected from all the experiments was analyzed separately for each parameter and subjected to two ways Analysis of Variance (ANOVA) using XL-STAT 2012. The means were compared for significance using Tukey’s HSD test and the values presented are mean of three replicates ± standard error (S.E).

**Results and discussion**

**Total phenols**

Phenolics are well-known antifungal, antibacterial and antiviral compounds occurring in plants that can accumulate in plants after infection [16]. These compounds indubitably play an important role in plant defense by enhancing the mechanical strength of host cell walls by the synthesis of lignin and suberin, which were involved in the formation of physical barriers that can block the spread of pathogens [17]. In present study, all the tested genotypes were susceptible to phytoplasma infection and there was no significant difference in the total phenols of healthy plants of all tested genotypes except Desi type Aug-424 (Figure 1). However, in phytoplasma-infected plants total phenols were significantly higher in leaves of Aug-424 while significantly lower in C-727 over their healthy control plants. However, there was no statistical difference in amount of total phenols in healthy and diseased plants of two Kabuli (Noor-2009 and CM-2008) and one Desi type (Bittle-98) genotype (Figure 1). The maximum amount of total phenols was observed in symptomatic plants of genotype Aug-424 and Bittle-98. These findings pointed out that on phytoplasma infection different chickpea genotypes respond differently in terms of defense through modulation in phenolic contents.

Generally high level of phenolics in plants after infection has been correlated with increased resistance [8] but few studies also showed that phenolic compounds are not only related to the resistance but also perform other functions in plant tissues [18]. A possible explanation for decrease in phenolic compounds in diseased plants of genotype C-727 is that many plant pathogens actively suppress the expression of plant defense reactions during successful infection [19].

**Protein content**

In present study, there was variable trend in the protein contents of healthy plants of all tested genotype (Figure 2). Maximum protein contents were observed in healthy plants of Desi type genotype C-727. Significant reduction in protein contents was observed in leaves of phytoplasma-infected plants of Noor-2009, Bittle-98 and C-727 while change was non-significant in Aug-424 as compared with healthy plants. On the other hand, significant increase in protein contents over their healthy plants was observed in leaves of phytoplasma-infected plants of Desi type.
genotype CM-2008. Maximum decrease in protein contents in leaves of infected plants as compared to healthy plants was observed in C-727 (Figure 2).

Involvement of proteins in plant disease resistance has been documented in many plant pathogenic interactions [20]. Usually infected plants show a high protein contents, which could be due to both the activation of the host defense mechanisms and the pathogen attack mechanisms. A similar trend was also observed in present findings regarding phytoplasma-infected plants of Desi type genotype CM-2008. A possible explanation for significant decrease in total soluble protein contents in some genotypes after infection in present study may be due to high level of susceptibility of these genotypes. Same kind of results has been reported in maize, tomato, grapevine and apple infected with other phytoplasmas [21–23]. Researchers hypothesized that total protein reduction in phytoplasma-infected leaves might have been due to the decrease in synthesis of ribulose-1,5-biphosphate carboxylase (RuBPC), the major soluble protein of the leaf; which (RuBPC) plays the role as storage protein [24].

Phenylalanine ammonia-lyase activity (PAL)

PAL is the primary key enzyme in the synthesis of the secondary, endogenous signaling molecule salicylic acid, which in turn activates the expression of a variety of PR genes [25]. In present study, significant difference in the PAL activity was observed in healthy plants of all tested genotype of both Kabuli and Desi type genotypes (Figure 3). Maximum increase in PAL activity in leaves of infected plants over their healthy plants was observed in CM-2008 while maximum decrease was observed in C-727 (Figure 3). PAL activity was significantly increased in leaves of phytoplasma-infected plants of both Kabuli type genotypes. However, PAL was decreased significantly in all Desi type genotypes over their healthy plants. Earlier studies showed that the activation of PAL and subsequent increase in phenolic content in plants is a general response associated with disease resistance. So, under present study; significant increase in PAL in Kabuli type genotypes tried to offer protection while decrease in Desi type genotypes rendered tissues more susceptible to phytoplasma infection. Previous studies confirmed that reduction of phenylpropanoid metabolism through inhibition of PAL activity in transgenic tobacco also rendered tissues more susceptible to Cercospora nicotianae [26]. The results of the present study indicated that PAL activity is an important part of disease resistance mechanism in chickpea.

Peroxidase activity (POX)

POX is one of the first antioxidant enzymes responding and providing fast defense against plant pathogens by participating in a variety of defense mechanisms [27]. POX is involved in the production of reactive oxygen species, which are directly toxic to the pathogen or indirectly reduce the spread of the pathogen by increasing the cross-linkage and lignification of the plant cell walls [28]. POX is required for the final polymerization of phenolic derivatives into lignin and also involved in suberization or wound healing [20]. Increased POX activity was observed in a number of resistant interactions involving plant pathogenic fungal and bacterial interactions [29]. POX activity was significantly variable in healthy plants of all tested genotypes. Maximum POX activity was observed in healthy plants of Kabuli genotype CM-2008 while minimum in Kabuli genotype Noor-2009 and Desi genotype Bittle-98. POX activity was significantly increased in leaves of phytoplasma-infected plants of Noor-2009 and Aug-424 while it was significantly decreased in CM-2008 and Bittle-98 but remained unchanged in C-727 compared to their healthy plants (Figure 4).

Pathogen infections can increase the level of certain enzymes within plants, including those that could attack cell wall of pathogens and those that create/maintain environments unsuitable for pathogens i.e. peroxidases and polyphenol oxidases [30]. Interestingly, in phytoplasma-infected plants total phenols and POD activity were significantly higher in leaves of Aug-424 that point out their co-involvement in disease susceptibility. However, this aspect needs to be further tested.
Catalase activity (CAT)

CAT is an oxygen-scavenging enzyme that removes toxic substrates (H₂O₂) during development, which are otherwise lethal [31]. CAT activity was different in healthy plants of all tested genotypes (Figure 5). Maximum CAT activity was observed in healthy plants of Kabuli genotype Noor-2009 while minimum in Desi genotype Bittle-98. CAT activity was significantly decreased in leaves of phytoplasma-infected plants of Noor-2009, Aug-424 and C-727 but non-significantly in CM-2008 and Bittle-98 over their healthy plants (Figure 5).

Present results agree with Hernandez et al. [32], who observed decreased CAT activity in two susceptible apricot cultivars infected with plum pox virus. They concluded that reduction in CAT activity could be a consequence of enhanced proteolysis caused by peroxisomal endopeptidases, which are induced by oxidative stress [33], as previously is in the case of Pyricularia oryzae infection in wheat [29]. Therefore, present findings further evidence for reduction in CAT activity in the leaves of all tested chickpea genotypes during infection. Moreover, the reduction of CAT activity usually increases plant resistance to pathogenic attack because plants can maintain high concentrations of H₂O₂ [34]. However, the role of CAT in the plant–pathogen interaction seems to be more complex than for abiotic stress [35] which involves an association between CAT activity and plant tolerance [36].

Polyphenol oxidase activity (PPO)

PPO activity was variable in healthy plants of all tested genotypes (Figure 6). Maximum PPO activity was observed in healthy plants of Kabuli genotype CM-2008 and Desi genotype Bittle-98 while minimum in Desi genotype C-727. PPO activity was significantly increased in leaves of phytoplasma-infected plants of Desi genotype C-727 but non-significantly in Aug-424. These results suggest that antioxidant enzymes can be activated in response to infection by phytoplasma. However, PPO decreased significantly in Noor-2009, CM-2008 and Bittle-98 compared to their healthy control plants (Figure 6).

PPO is an important enzyme in the initial stage of plant defense where membrane damage causes release of phenols such as chlorogenic acid. PPO catalyzes the oxidation of phenolics to free radicals, which can react with biological molecules, thus creating an unfavorable environment for pathogen development [37]. According to researchers, antioxidative enzymes (POX and PPO) may participate in the defense reaction by inducing plant resistance against pathogenic agents [38]. Our results are in agreement with the previous finding [39] showing a correlation between increased antioxidant enzyme activities and pathogen resistance in plants.

Enzymes involved in phenol metabolism were mostly considered as one of the important biochemical parameters for disease resistance. Polyphenol oxidases catalyzes the oxidation of monophenol and o-dihydroxy phenol [40]. The enhanced polyphenol activity in Aug-424...
and C-727 might have resulted in the augmented rate of oxidation of phenolics substance that participates in the defense reaction of host.

**Chlorophyll pigments**

Chlorophyll (Chl) contents were different in healthy plants of all tested genotypes. Healthy plants of all Desi genotypes showed higher amount of Chl a and total Chl contents as compared to both Kabuli genotypes (Figures 7 and 8). However, Chl b contents in healthy plants of all tested genotypes differ significantly irrespective of their classes i.e. Kabuli and Desi (Figure 8). Maximum Chl a, Chl b and total Chl were observed in healthy plants of Desi type genotype Aug-424 (Figures 7 –9).

Phytoplasma infected plants of all tested genotypes showed a significant decrease in the concentration of Chl a, Chl b and total Chl contents as compared to uninfected plants depending upon the genetic make-up of the genotypes (Figures 7–9). Reduction in Chl a contents of infected leaves was less in both Kabuli genotypes as compared to Desi type genotypes over their healthy plants. While maximum reduction in Chl b was observed in phytoplasma infected plants of Desi type genotype C-727. Maximum decrease in total Chl contents was observed in phytoplasma infected plants of both Kabuli type genotypes while minimum in Desi genotype Bittle-98. In present study, Chl b was found to be more sensitive to phytoplasma infection than Chl a as Chl b was more affected than Chl a by the deleterious toxins secreted by phytoplasma.

Several studies reported that carbohydrate metabolism variation in plants that are affected by phytoplasmas, correlates with a marked reduction of total Chl content due to decrease of both Chl a and Chl b in leaves [41]. The reduction in chlorophylls shows that the phytoplasma can interfere in photosynthesis. The marked reduction of total Chl in phytoplasma infected leaves in present study, may be due to the decrease of both Chl a and Chl b contents as previously reported in case of many viruses and phytoplasma infected plants. Present findings also supported the findings of researchers who suggested that phytoplasmas have a role in the inhibition of chlorophyll bio-synthesis in plant host leaves [22]. Numerous studies have revealed that pathogen infections significantly reduced the Chl a and Chl b contents [42]. As in present investigation, Chl a and Chl b were degraded, it can be decided that phytoplasma infection might affect chlorophyll molecules by degradation or through inhibition of synthesis of both Chl a and Chl b molecules especially in genotypes more sensitive to disease. Chl a is believed to be the more exact characteristic of photosynthetic activity of plants [6]. Researchers suggested that reduced level of Chl, particularly Chl a is associated with the reduced rate of photosynthesis [43]. From present findings it can be assumed that the observed reduction in chlorophyll levels will probably interfere with the photosynthetic capacity in the chickpea leaves as previously observed for the papaya.
dieback and corn infected with maize bushy stunt phytoplasma pathosystems [18].

Conclusion

The present finding indicates that phytoplasma infection causes non-specific, general stress response in chickpea leaves. Alterations in level of phenolic compounds, total soluble proteins, PAL, POX, CAT, PPO, Chl a, Chl b, and total chlorophyll in leaves of phytoplasma infected plants might severely damages the physiological and biochemical processes which leads to the appearance of epidemiological symptoms characteristic to specific host-phytoplasma interaction. In conclusion, present study provided significant insights for better understanding the mechanisms of plant response to pathogen infection especially phytoplasma in chickpea.

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