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DETERMINATION OF ENDOGENOUS HORMONE LEVELS IN ASCOCHYTA BLIGHT [*Ascochyta rabiei* (Pass.) Labr.] SUSCEPTIBLE AND RESISTANT CHICKPEAS (*Cicer arietinum* L.)

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ABSTRACT

Ascochyta blight, caused by *Ascochyta rabiei* (Pass.) Labr., is the most important foliar disease of chickpea (*Cicer arietinum* L.) in many countries. Many studies have been carried out on basis of population biology and host-plant resistance of *A. rabiei*, but the effect of endogenous plant hormone levels on resistance to ascochyta blight of chickpea has been studied rarely. Therefore, the study was designed to compare endogenous plant hormone levels in ascochyta blight susceptible and resistant chickpea genotypes under ascochyta blight infected conditions. ILC 263, (susceptible to ascochyta blight), FLIP 95-60C and FLIP 98-224C (resistant to ascochyta blight) were used to determine the level of plant hormones; indole-3-acetic acid (IAA), zeatin, gibberellic acid 3 (GA_3), and abscisic acid (ABA). Concentrations of IAA, zeatin, GA_3 and ABA were markedly increased in pods of resistant genotypes, FLIP 95-60C and FLIP 98-224C. It was suggested that high zeatin and GA_3 concentrations in pods could be detected as biochemical markers to determine resistance to ascochyta blight of chickpea genotypes since the genotypic effect was statistically significant only for zeatin and GA_3. Considering the statistically significant genotypic effects only for zeatin and GA_3, these hormones may be used as biochemical markers to determine resistance to ascochyta blight of chickpea genotypes.

Key words*: *Kabuli chickpea; IAA; Zeatin; GA_3; ABA

INTRODUCTION

Blight of chickpea (*Cicer arietinum* L.), caused by *Ascochyta rabiei* (Pass.) Labr., is one of the most important foliar disease throughout the world (Bayraktar *et al*., 2007; Ahmed *et al*., 2009; Atik *et al*., 2011). Yield losses from disease have been reported in 35 countries (Nene *et al*., 1996). It was reported that the blight pathogen has both asexual and sexual stages with two different spore types (Kaiser *et al*., 1994). Wilson and Kaiser (1995) and Trapero-Casas and Kaiser (1992b) pointed out that the teleomorph of *Ascochyta rabiei* is known as *Didimella rabiei* (Kovachevski) v. Arx. (syn. *Mycosphaerella rabiei* Kovachevski). Pathogenic variability of *A. rabiei* has been demonstrated since the 1960s (Aujla, 1964; Kaiser, 1973; Grewal, 1984; Nene and Reddy, 1987; Kaiser, 1997). However, there has been no consensus as to whether the variability of *A rabiei* is due to race or aggressiveness in a single race (Mmbaga, 1997). Several researchers reported that there were six races of the disease (ICARDA, 1993; Singh and Reddy, 1993). The later works showed that there were thirteen different virulent isolates (Mmbaga, 1997). Kaemmer *et al*. (1992) applied oligonucleotide fingerprinting and DNA amplification fingerprinting for the molecular characterization of six isolates of *A. rabiei* and distinguished three groups. Recently *A. rabiei* isolates were classified into 3 groups (Pathotype-1, Pathotype-2 and Pathotype-3) based on the reactions to set of the host cultivars. Pathotype-3 is the most virulent of these three types (ICARDA, 1998; Udupa *et al*., 1998; ICARDA, 1999; Jamil *et al*., 2000). Similarly, it was pointed out that 48 isolates from different countries were grouped into 5 groups using RAPD markers (Santra *et al*., 2001). In a genetic diversity study in Turkey, 64 isolates from different geographical origin were grouped into 7 groups using UPGMA based on microsatellite-primed-PCR but the groups did not matched with the geographical origin (Bayraktar *et al*., 2007).

Although it is possible to control of ascochyta blight by the use of such inputs as agricultural chemicals, economic and environmental concerns widely restrict their use by farmers (Nene and Reed, 1994). The most economic and practical control of diseases can be achieved through integrated management systems, including host-plant resistance and improved agricultural practices (Singh, 1997). Cagirgan *et al*. (2011) suggested that high level of malic acid may be used as pre-selection criteria for resistance to ascochyta blight in chickpea

breeding material but the effect of endogenous plant hormone levels on resistance to ascochyta blight of chickpea has not been studied so far. Therefore, this study focuses on determination of endogenous plant hormone levels in susceptible and resistant genotypes of chickpea, subjected to *A. rabiei*.

MATERIALS AND METHODS

Plant materials

There are two main types of cultivated chickpeas (*Cicer arietinum* L.). The small-seeded desi and large-seeded kabuli types (Auckland, 1977). Three kabuli chickpea lines, ILC 263 (susceptible to ascochyta blight), FLIP 95-60C and FLIP 98-224C (resistant to Ascochyta blight) from the International Center for Agricultural Research in the Dry Areas (ICARDA) were used. Genotypes were sown in the first week of December in 2000 and the second week of December 2001 at the experimental station of Akdeniz University in Antalya, Turkey. Genotypes were grown in a randomised complete blocks design with two replications. The experimental plots consisted of one row of 4 m length with inter and intra row distances of 45 x 10 cm. The susceptible check, ILC 263, was repeated in each two-test row as a spreader-indicator row in order to enhance epidemics. The experimental area was hand weeded.

Antalya has mild and wet winters, and hot and dry summers. Monthly and seasonal distribution of precipitation is typical of Mediterranean climates. In the experimental area, generally organic matter and macro plant nutrients were found at low levels with 0.1% total nitrogen. Soil texture of experimental area was loam with a pH of 8.05.

Inoculation of genotype

Several breeders or pathologists have focused on ascochyta blight and pointed out that the fungus survives in the diseased chickpea debris and in seeds from infected plants (Maden *et al*., 1975; Maden, 1983; Nene, 1984; Kaiser and Hannan, 1988; Trapero-Casas and Kaiser, 1992a). Infected debris is an important source of infection in the following seasons because the fungus survives for two years in infected tissues. In this study, we used infected debris for inoculation of the plants. The previously collected infected tissues were dispersed on the plants during the initial flowering and pod filling stages as reported by Singh *et al*. (1981; 1984) and Muehlbauer *et al*. (1998).

Disease assessment

Most breeding programs are based on visual scoring in greenhouse or field conditions. Many breeders have developed visual scoring for breeding programs, *e.g*. Vir *et al*., (1975); Reddy and Singh (1984); Reddy *et al*. (1984); Haware *et al*. (1995). According to Toker *et al*. (1999), disease rating scale was scored by using 1-9 class scale, where 1 = Immune, 5 = Tolerant and 9 = Very Highly Susceptible (all plants killed by disease). Scoring was taken after pod filling stage.

Hormone analyse

After genotypes were evaluated for their reaction to ascochyta blight for two years, they were used for hormone analyses in the second year. The whole youngest fully emerged leaf, with leaflets and rachis, and green pod with immature seeds were used. Harvesting was done during the second week of May in 2002, while plants were at the early pod filling stage. After excising plant organs with scissors without touching them by hand in the evening, they were placed in nylon boxes and then frozen. One-gram samples of leaf or green pod with immature seeds were homogenized in cold methanol: chloroform (14:6 v/v) mixture at room temperature, and then they were stored at -20 ^{o}C for one week. The extracts were filtered through Whatmann No 5 filter paper and the residue re-homogenized with the same solution mixture, and the extracts were combined. The aqueous residue was adjusted to pH 8.5 with 1 N NaOH, and transferred to a separating funnel to separate chloroform from the methanol. The chloroform phase was discarded. The methanol phase was reduced at 40 ^{o}C to an aqueous phase under reduced pressure on a rotary evaporator. It was then adjusted to pH 2.5 with 1 N HCl and extracted with ethyl acetate (3 volumes). The aqueous phase was adjusted to pH 7 with 1 N NaOH and extracted with ethyl acetate (3 volumes), and then the acidic and neutral ethyl acetate phases including free hormones were combined. In order to release conjugated hormones, the aqueous phase was adjusted to pH 11 with 1 N NaOH and incubated in a water bath at 70 ^{o}C for one hour. The hydrolysate was adjusted to pH 7 with 1 N HCl and extracted with ethyl acetate (3 volumes), and then the aqueous phase adjusted to pH 2.5 with 1 N HCl, extracted with ethyl acetate (3 volumes). Acidic and neutral ethyl acetate phases were combined, and then this combined hormone conjugated extract was combined with the previous free hormone extract and reduced to dryness in vacuum at 40 ^{o}C. The residue was dissolved in 1 ml methanol, and transferred to a microcentrifuge tube. The methanol was reduced to 100 µl under vacuum, and then line-loaded onto a 20 X 20 cm, 0.25 mm thick silica gel 60 F_{254} TLC plate (Merck Plc, Darmstadt, Germany). Standard ABA, IAA, GA_3 and zeatin were also spot-loaded in scored strips at both edges of the plates. The plate was allowed to develop for 15 cm in the vertical direction using methanol: ammonia: water (84:8:8 v/v) as the solvent system. After development, the position of ABA, IAA, GA_3 and zeatin were detected under UV light (254 nm wave-length) and marked. A band of silica corresponding to the Rf values of standards was scraped off, dissolved in 0.5 ml methanol in a microcentrifgue tube, and then dried under vacuum. The purified samples methylated with diazomethane (Schlenk and Gellerman, 1960) dissolved in ethyl ether and methanol (9:1 v/v). The derivatives were dried under vacuum and re-dissolved in 100 µl ethyl acetate for GC analysis. Levels of the ABA, IAA, GA_3 and zeatin were

determined with a Fisons 8560 HRGC Mega 2 series equipped with FID, and using a SPB-1 (30 m x 0.32 mm I.D.) capillary column. Injection and detector temperatures were 200 ^{o}C and 300 ^{o}C, respectively. Samples (1 µl) were injected into the column at 80 ^{o}C. The temperature was programmed 5 ^{o}C min^{-1} until the column was at 280 ^{o}C. The flow of the He gas was maintained 22 p.s.i. / min.

Statistical analysis

Recorded data were analysed by using MSTATC package program (Freed *et al*., 1989). Susceptible *vs* resistant groups were compared statistically by using orthogonal contrast comparison feature of the software for all the traits studied.

RESULTS

Reaction to ascochyta blight

FLIP 95-60C and FLIP 98-224C, scored 2 for *Ascochyte* blight symptoms, *i.e*. resistant over two years under field conditions. As expected, the susceptible genotype, ILC 263, scored more than 8 on 1-9 class scale over two years. Especially in the second year, ILC 263 was killed by the pathogen, *Ascochyta rabiei*, in the all rows, except several plants in one row.

Plant hormones

Analysis of variance showed that genotype effects were significant ($p \leq 0.05$) for IAA and zeatin in leaves ($p \leq 0.01$), but not significant for GA_3 and ABA in leaves. In green pods with immature seeds, it was found that there were statistically significant differences ($p \leq 0.05$) among genotypes for zeatin and GA_3, but not for IAA and ABA ($p > 0.05$). Furthermore, orthogonal contrast comparisons revealed that there were statistically significant differences between resistant and susceptible genotypes for IAA, zeatin and ABA concentrations in leaves; and zeatin and GA_3 levels in pods ($p \leq 0.05$).

IAA, zeatin, GA_3 and ABA concentrations in leaves are shown in Fig.1 and Fig. 2.

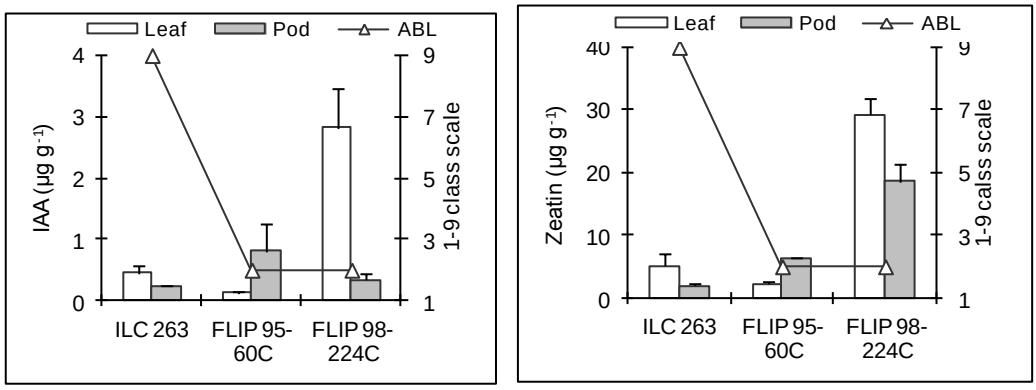

Figure 1. Mean values of IAA and zeatin (µg g^{-1} fresh weight) in fully expanded leaf and green pod with immature seeds during pod filling stage of kabuli Chickpeas, susceptible and resistant to ascochyta blight when genotypes subjected to *A. rabiei*. ABL is ascochyta blight score on a 1-9 class scale. Values are means ± standard deviations.

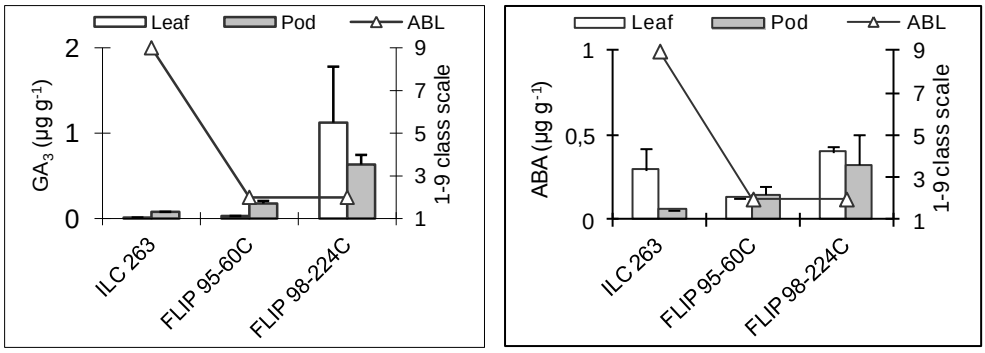

Figure 2. Mean values of GA_3 and ABA (µg g^{-1} fresh weight) in fully expanded leaf and green pod with immature seeds during pod filling stage of kabuli chickpeas, susceptible and resistant to ascochyta blight when genotypes subjected to *A. rabiei*. ABL is ascochyta blight score on a 1-9 class scale. Values are means ± standard deviations.

Mean concentrations of IAA and zeatin in leaves of genotypes changed from genotype to genotype. Likewise, ABA accumulation in leaves of genotypes displayed the same pattern. IAA levels in leaves ranged from 0.13 µg g^{-1} FW in FLIP 95-60C to 2.81 µg g^{-1} FW in FLIP 98-224C.

Similarly, zeatin concentrations of leaves changed between 2.20 µg g^{-1} FW in FLIP 95-60C and 29.30 µg g^{-1} FW in FLIP 98-224C. On the contrary, GA_3 level in leaves of genotypes ranged from 0.01 µg g^{-1} FW in ascochyta blight susceptible genotype ILC 263, to 1.13 µg g^{-1} FW in FLIP 98-224C (resistant to ascochyta blight). Also, the

GA_3 level in leaves of FLIP 95-60C was higher than in the susceptible genotype ILC 263. ABA accumulation in leaves ranged from 0.13 μg g^{-1} FW in FLIP 95-60C to 0.40 μg g^{-1} FW in FLIP 98-224C. ILC 263 had ABA values of 0.30 μg g^{-1} FW in leaves and 0.06 μg g^{-1} FW in pods.

As can be seen in Fig.1 and 2, all of the plant hormones studied were higher in pods of resistant genotypes than of the susceptible one. IAA concentrations in pods ranged from 0.25 μg g^{-1} FW in susceptible genotype (ILC 263) to 0.81 μg g^{-1} FW in FLIP 95-60C, resistant to ascochyta blight. The other resistant genotype, FLIP 98-224C, had an IAA concentration of 0.34 μg g^{-1} FW. The resistant genotype, FLIP 98-224C, had the highest level of zeatin, GA_3 and ABA in pods (Fig. 2). The all endogenous hormone accumulations in pods of the resistant genotypes were higher than in the susceptible one (Fig 1 and 2).

DISCUSSION

A. rabiei isolates were classified into 3 groups (Pathotype-1, Pathotype-2, Pathotype-3) based on the reactions to set of differentials (ICARDA, 1999). Pathotypes used in the study may be Pathotype-1 and Pathotype-2, since resistant genotypes, FLIP 95-60C and FLIP 98-224C, were rated as resistant with rating 2 on 1-9 class scale since the Pathotype-3 is known as the most aggressive one among the pathotypes.

Although total hormone concentrations in the resistant genotypes were higher than in the susceptible genotype, ILC 263 as apparently seen from Fig. 1 and 2, plant hormones could be affected by environment and also changed different organs of the same plants (e.g. Davies, 1995). In general, average hormone levels increased in leaves with exception. Specifically, in resistant genotype, FLIP 98-224C, IAA and zeatin increased two-fold in leaves than pods. It is shown that leaves of resistant genotypes are less affected by *A. rabiei*. IAA, zeatin and ABA concentrations in leaves of FLIP 95-60C, the other resistant genotype, were lower than in the susceptible genotype, ILC 263. The GA_3 concentration in leaves of FLIP 95-60C was slightly higher than in ILC 263. FLIP-98-224C had significantly higher GA_3 concentration than ILC 263. Kaur *et al*. (1998) reported that GA_3 reversed the effect of salt stress in chickpea seedlings by enhancing amylase activity and mobilization of starch in cotyledons. Also, exogenous application of zeatin promoted floret development and increased the number of fertile florets as well as grain set in wheat (*Triticum aestivum* L.). In contrast, IAA, GA_3 and ABA inhibited floret development in wheat (Wang *et al*., 2001). Jameson (2000) suggested that both cytokinins and auxins were involved in symptom development in plants. Li *et al*. (2000) studied the expression and distribution of zeatin O-glycosyltransferases in different tissues from maize and bean under cold stress.

In conclusion, high zeatin and GA_3 concentrations in pods suggest that these hormones may be considered as biochemical markers to differentiate resistant and susceptible chickpea genotypes for ascochyta blight when chickpea genotypes are subjected to the disease. A detailed correlation study with more entries of breeding lines remains a further task in order to determine indirect selection criteria via endogenous hormones studied here for resistance to the ascochyta blight.

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