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Determination of endogenous zeatin/IAA levels in selected fortune mandarin mutants against *Alternaria alternata* pv. citri

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ABSTRACT

Alternaria brown spot is a serious disease in mandarins and mandarin hybrids. In this particular research, 9 Alterneria tolerant and 2 susceptible mutant individuals obtained from a series of *in vitro* and *in vivo* studies were used. Endogenous indole acetic acid (IAA) and zeatin hormone levels of the individuals were noted before and after the Alternaria disease inoculations in order to determine the hormonal variations during the research. It was determined that endogenous zeatin levels decreased after the inoculation compared to its initial levels. The indole acetic acid levels of the individuals increased after inoculation except for some individuals.

1. Introduction

Alternaria brown spot disease is caused by a necrotypic fungus *Alternaria alternata pv.* citri. Alternaria brown spot was found for the first time in Australia on the 'Emperor' tangerines cultivar in 1903 (Pegg 1966). Since then the disease has been found in many countries in different continents including South Africa (Schutte et al. 1992), Israel (Solel 1991), Cuba (Herrera 1992), Colombia (Castro Caicedo et al. 1994), Turkey (Canıhoş et al. 1997), Argentina (Peres et al. 2003) and Peru (Marin et al. 2006). Alternaria brown spot is an important disease because of its effects on leaves, branches and unripe fruits of the tangerine and its hybrids (Pegg 1996; Canıhoş et al. 1999). Among tangerine cultivars and their hybrids, particularly 'Dancy' and, to a lesser extent, 'Fortune' are the most susceptible to the disease (Nemsa et al. 2012). Similarly, Peever et al. (2000) reported that 'Minneola', 'Orlando', 'Sunburst' and 'Nova' hybrids were also very sensitive to this pathogen. In the meantime, the toxic substance secreted by the same pathogen was found to be effective on mandarin 'Dancy' cultivar and its hybrids as well as mandarin x grapefruit hybrids and mandarin x orange hybrids (Vicent et al. 2007).

Nowadays, Alternaria brown spot disease is considered to be the most detrimental fungal disease on tangerine and its hybrids. This particular disease causes serious problems especially for the late season mandarin cultivars such as Minneola tangelo and Fortune in Turkey.

A number of interior and environmental factors, which work together in complex synergisms and antagonisms, regulates resistance responses of plants to the disease. The plant growth substances have vital importance among these factors (Pieterse et al. 2009; Santner et al. 2009; Jaillais and Chory 2010). The interactions between salicylic acid and jasmonic acid/ethylene (SA-JA/ET) are accepted as the backbone of immunity in plants (Pieterse et al. 2012). These hormones are considered to be stress hormones (Baktır 2015). However, traditional plant growth regulators such as auxins, gibberellic acids, cytokines and abscisic acid protect the plants against invasive hazardous pathogens or increase the immunity systems of the plants (Pieterse et al. 2012; Naseem et al. 2012).

Skoog and Miller (1957) described opposite behaviors of auxins and cytokinins in root and shoot developments of these plants, respectively. Consequently, auxins are accepted as rooting and cytokinins as shooting hormones (Baktır 2015). Auxins suppress the response of salicylic acid on plant immunity systems and this situation partially strengthens the role of jasmonic acid (Robert-Seilaniantz et al. 2011; Naseem and Dandekar 2012). Naseem et al. (2012) determined that external cytokinin applications prevented the development of the pathogens from the research they conducted on the interaction between cytokinins and salicyclic acid related to SA-biosynthesis in the mutants (sid2). This finding showed that cytokinin signals increase the resistance or immunity of the plants in comparison with salicyclic acid inductions in hormone/disease networks.

Cytokinins come into interaction with salicyclic acid sensitivity factor TGA3 to activate the transcriptional regulator ARR2 (*Arabidopsis* response regulator) which promotes salicyclic acid stimulation (Choi et al. 2011). Therefore, cytokinins can act synergistically on the salicyclic acid excitation pathway (Galis et al. 2004).

In this research, the variations in levels of auxin and zeatin in 9 Alternaria resistant (M_1V_3) and 2 Alternaria sensitive (M_1V_3) Fortune mandarin mutants were determined both before and at

the end of the research (Table 1). The mutants used in this research were obtained through the artificial irradiation method.

Table 1. The sensitivity of Fortune mandarin mutants to the Alternaria brown spot disease

Genotype	Tolerance
7-4-1	Tolerant
1A	Tolerant
2A	Tolerant
1-4-1	Tolerant
2B	Tolerant
6B	Tolerant
4-3-6	Tolerant
5-3-2	Tolerant
6D	Tolerant
3A	Susceptible
5-3-5	Susceptible

2. Materials and Method

After the application of acute gamma rays of 50 and 60 gray doses into "Fortune" budwoods, the radiated budwoods were grafted onto common sour oranges (M_1V_1). Following the grafting, the plants were vegetatively grown and brought to M_1V_2 and M_1V_3 stages. In this particular stage, 9 tolerant mutant individuals (M_1V_3) and 2 sensitive mutant individuals (M_1V_3) were used (Turgutoğlu and Baktır 2019).

The genotypes were carefully pruned in order to encourage the growth of new shoots and leaves. The fungus culture were conducted as described by Diaz et al. (2018). Following the pruning, when new leaves reached to lengths of 1 to 3 cm, each leaf was inoculated with an Alternaria spore suspension containing $5x10^5$ spores per ml (Azevedo et al. 2010). Perez-Jimenez and Perez-Tornero (2021) reported that the application of the toxin to the excised and wounded leaves seemed to be the most reliable method among the test methods to analyze sensitivity to Alternaria of 'Fortune' explants cultured *in vitro*. Following the inoculation, the plants were transferred into polyethylene bags in order to preserve the humidity and prevent the leaves drying out. According to Dalkilic et al. (2005) symptoms of the disease usually appear 24 hours after the inoculation. For this reason, symptoms of the disease were observed and examined at the indicated time on the inoculated plants. Physical conditions for the plants were adjusted to 26±2°C and 80-85% humidity rate in the growing rooms during the research.

Extraction and chromatographic analyses were done in the leaves before both the inoculation and 24 hours after the inoculations when the signs of disease appeared on the leaves in order to determine the variations of endogenous auxin and cytokinin levels in the plants. The extraction and purification processes were made in accordance with Kuraishi et. al. (1991), Battal and Tileklioğlu (2001), Erez (2009) and Atmaca (2015).

Extractions of the sampled leaves were chopped for 10 minute in a homogenizer. The leaf samples were treated with 80% methyl alcohol at 4°C before the homogenization. The homogenized material was kept for 24 hours at 4°C in the dark.

The residue remaining on the filter was discarded while the aqueous portion was removed after the samples were filtered through Whatman No: 1 filter paper. The methyl alcohol remaining in aqueous portion was evaporated at 45°C through the evaporator. The extract freed from methyl alcohol and dissolved with 0.1 M KH_2PO_4 (pH 8.0) was taken from the round bottom flask and centrifuged at 6000 rpm for an hour at 4°C.

The samples were placed into a beaker by discarding the sediment part in the tubes and were shaken for 1-2 minutes in order to separate phenolic and colored compounds after adding 1 gr PVPP (polyvinyl poly pyrrolidone, Sigma) (Erez 2009). It was then filtered through Whatman No: 1 filter paper. The cartridge was conditioned by passing 2.5 ml of methanol (80%) and 2.5 ml of distilled water through the filtrate cartridge before applying the filtrate to the Sep-Pak C 18 Cartridge. After this application, the filtrate was passed through the cartridge to keep the hormones in the cartridge. The derived hormones adsorbed in the cartridge were taken into vials using 5 ml of methanol (80%), and then injected into High Performance Liquid Chromatography (HPLC, LC 20 AT model). IAA and Zeatin analysis were done in HPLC according to Morris et al. (1990).

The following analysis systems were applied during the hormone analysis in HPLC:

Detector-DAD (Diode Array Detector, SPD–M20A)

Column-Inerstil C_{18} (5 µm, 250 x 4.6, GL Science, Tokyo, Japan)

Floe rate: 1 ml min^{-1}

Mobile phase: Methanol and 0.1 M acetic acid (55/45 v/v) (Ülger et al. 1999)

Wavelengths: 276 nm for IAA and 272 nm for Zeatin

The experimental data were statistically analyzed with the general linear model (GLM). Means were compared using LSD's Multiple Range Test.

3. Results and Discussion

In the study, 9 tolerant and 2 susceptible individuals from Fortune mandarin mutants were used for both *in vivo* and *in vitro* evaluations (Table 1).

It was determined that the zeatin level decreased in disease inoculated types except 3A genotype after the inoculation compared to pre-inoculated ones. The highest zeatin level was determined in 6D genotype with 3.48 ppm before the inoculation. The level of zeatin decreased to 2.71-ppm level in the same genotype after the inoculation. The lowest zeatin level with 0.58 ppm was found in 4-3-6 tolerant genotype after the disease inoculation. In general, zeatin levels were found to have decreased after the disease inoculations compared to pre-disease inoculations (Table 2).

IAA levels decreased in five of the genotypes (7-4-1, 2A, 1-4-1, 2B and 6B) obtained through mutations compared to pre-inoculated ones. On the other hand, IAA levels increased in genotypes 1A, 4-3-6, 5-3-2, 6D and 5-3-5 after the inoculations. The highest level of IAA was detected in genotype 5-3-2 with 2.12 ppm after the inoculation while the lowest IAA level was detected in genotype 7-4-1 with 0.04 ppm after the inoculation (Table 3).

The individual roles of auxin and cytokinin differ according to the plant and pathogen systems (Navarro et al. 2006; Wang et al. 2007; Choi et al. 2010; Choi et al. 2011). Kazan and Manners (2009) conducted research on *Arabidopsis* by inoculating *Pseudomonas syringae pv. tomato* DC3000 (Pto) in order to find out possible effects of auxins in *Arabidopsis*. They reported that auxins in general increased plant sensitivity and suppressed PR1 genes, related to increasing auxin levels. Despite this report,

Table 2. Changes in endogenous zeatin (Z) levels in Fortune mandarin mutants before and after the inoculations with *Alternaria alternata pv. citri*.

Genotypes	Zeatin (Z) levels (ppm)		Average Z value of the genotypes
	Before inoculation	After inoculation	
7-4-1	1.36ı*	0.60pq	0.98 ± 0.144h
1A	1.36ı	0.42r	0.89 ± 0.178ı
2A	1.92f	0.64p	1.28 ± 0.242f
1-4-1	1.10l	0.22s	0.66 ± 0.167j
2B	1.99e	1.50h	1.75 ± 0.093d
6B	1.85g	0.99n	1.42 ± 0.163e
4-3-6	3.06b	0.58q	1.82 ± 0.469c
5-3-2	1.06lm	0.88o	0.97 ± 0.036h
6D	3.48a	2.71c	3.10 ± 0.146a
3A	1.17k	2.36d	1.77 ± 0.225d
5-3-5	2.71c	1.33ı	2.02 ± 0.261b
Fortune	1.24j	1.03mn	1.14 ± 0.036g
Average value of the applications	1.86 ± 0.121A	1.11 ± 0.117B	

*The differences are statistically important between different letters (P<0.05). LSD (0.05), Genotype: 0.0355, Application: 0.0145, Genotype x Application: 0.0502.

Table 3. Changes in endogenous IAA levels in Fortune mandarin mutants before and after the inoculations with *Alternaria alternata pv. citri*.

Genotype	IAA levels (ppm)		Genotype average
	Before inoculation	After inoculation	
7-4-1	0.63l*	0.04r	0.34 ± 0.112j
1A	0.25p	0.54m	0.40 ± 0.056ı
2A	2.05b	1.50f	1.78 ± 0.105a
1-4-1	0.38o	0.05r	0.22 ± 0.063k
2B	1.04h	0.89ı	0.97 ± 0.031f
6B	1.99c	0.21p	1.10 ± 0.337e
4-3-6	0.48n	0.72k	0.60 ± 0.047h
5-3-2	1.23g	2.12a	1.68 ± 0.169b
6D	0.14q	1.48f	0.81 ± 0.254g
3A	0.84ıj	0.80j	0.82 ± 0.014g
5-3-5	0.56m	1.85d	1.21 ± 0.244d
Fortune	0.83j	1.68e	1.26 ± 0.036c
Application averages	0.87 ± 0.095B	0.99 ± 0.105A	

*The differences are statistically important between different letters (P<0.05). LSD (0.05), Genotype: 0.0355, Application: 0.0145, Genotype x Application: 0.0502.

a number of researchers indicated that high levels of cytokinin activated PR1 genes and induced increments of gene resistance (Naseem et al. 2012; Choi et al. 2010; Choi et al. 2011). It has been known that higher zeatin levels increase resistance of plants against some viral diseases and harmful insects (Ballare 2011).

Auxin was analyzed and tested by Kazan and Manners (2009) to identify its effect on endurance dynamic interactions of plant pathogens. It was proven that *Pseudomonas syringae pv. tomato* DC3000 (Pto) increased auxin biosynthesis during its infection period in tested plants (Chen et al. 2007). Meantime, it was reported that the roles of phytohormones auxin and cytokinin were independent in plant immunity (Robert-Seilaniantz et al. 2011). In another study, the same researchers show that Pto increased auxin accumulation and decreased cytokinin levels relative to baseline levels (Robert-Seilaniantz et al. 2011).

Considering the results obtained in this study, significant differences were detected in both zeatin and auxin levels between genotypes before and after disease inoculation. The IAA levels increased in most of genotypes after disease infections while zeatin levels decreased. There seems to be many important dynamics in the growth and development of plants related to interactions between auxin and cytokinin. Nevertheless, there is a combination of different hormonal networks as well as auxin and cytokinin concerning plant resistance to the disease infections in Fortune mandarin.

4. Conclusion

In this particular research, 9 *Alterneria alternata pv. citri* tolerant and 2 susceptible mutant individuals obtained from a serious of *in vitro* and *in vivo* studies were inoculated with the disease. The IAA levels increased in most of the genotypes after disease infections, while/whereas zeatin levels decreased. The differences in hormone levels likely occurred in mutant individuals due to some possible changes in their genetic structures.

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