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Phenotypic and Molecular Screening of Apple Genotypes to Woolly Apple Aphid Resistance

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Abstract

Woolly apple aphid (WAA) is a major pest of apple orchards resulting in significant losses. In the present study, 22 apple (*Malus domestica* Borkh.) cultivars were tested in the filed for their relative resistance/susceptibility to WAA. These apple cultivars were found to respond differentially to WAA infestation. Based on tree infestation rating, cultivars were ranked into 6 relative resistance/susceptibility groups, as follows: immune - 'Golden Delicious', 'Delbarestivale', 'Golden Smoothie', 'Red Miracle' and 'Harmony'; resistant - 'Stark Gold', 'Early Gold' and 'Argi Gala'; moderately resistant - 'Evanee' and 'Black Stayman'; moderately susceptible - 'Vista Bella', 'Jonagold', 'Royal Gala', 'Jersey Mac', 'Granny Spur Type' and 'Summerred'; susceptible - 'Jonathan', 'Nagava 6', 'Florina', 'Red Chief' and 'Gold Iralis'; highly susceptible - 'Fuji 6'. Eight molecular markers linked to major WAA resistant genes (*Er1*, *Er2*, and *Er3*) were screened in apple cultivars using PCR. The markers NZms_EB145764, NZms_EB106753 and NZsc_E01 were ubiquitous in all cultivars under study, whereas, NZsn_O05 was absent. The results of other markers revealed distinct patterns of amplification among apple cultivars. No clear correlations can be made between the molecular data (marker presence and absence) and the phenotypic results (cultivar ranking). The differences among cultivars regarding WAA infestation can potentially be utilized by apple breeders and commercial growers to achieve effective, environmental-friendly, and low-cost pest control.

Keywords: cultivar, *Eriosoma lanigerum*, host plant resistance, infestation rating, *Malus domestica*, resistance, susceptibility

Introduction

Apple (*Malus domestica* Borkh.) is one of the most economically important fruit crops of the temperate zones of the world (Harris *et al*., 2002). Apple trees are usually attacked by several types of arthropod pests (Shoonhoven *et al*., 2005). Aphids (order Homoptera) are key pests in apple orchards worldwide (Bloomers, 1994; Prokopy and Croft, 1994; Beers *et al*., 2003). Aphid infestation in apple causes deformation of foliage and fruits. In addition, some aphid species can act as vectors for virus transmission and the release of their honeydew promotes the development of mold infestation (Beers *et al*., 2003; Blommers *et al.,* 2004; Arbab *et al*., 2006).

The woolly apple aphid (WAA), *Eriosoma lanigerum* (Hausmann) (Hemiptera: Aphididae), is one of the most important aphid pests of apples in many of the world's apple growing regions (Hatton, 1937). WAA is becoming critical to the economics of apple industry in Jordan (Ateyyat and Antary, 2009; Ateyyat and Antary, 2010). WAA colonizes both the roots and the vegetative parts of apple trees (Alspach and Bus, 1999). This aphid pest overwinters in edaphic colonies on roots and produces its first instar nymphs (crawlers) in early spring (Thwaite and Bower, 1983). In spring, crawlers migrate upward and initiate new colonies in protected arboreal parts of the tree (Heunis and Pringle, 2006; Cockfield and Beers, 2007).

WAA infestation on roots resulted in the formation of hypertrophic galls which restricted water and nutrients uptake (Brown and Schmitt, 1990; Brown *et al*., 1995). Strong WAA infestations resulted from population outbreaks in the arboreal part of the tree, and can lead to reduction in canopy vigor due to premature defoliation, reduction in yield due to reducing fruit buds and splitting of fruit bearing wood, and reduction in fruit quality as a result of contaminating fruits with honeydew and sticky wool (Bertus, 1986; Brown and Schmitt, 1990; Brown *et al*., 1995; Pringle and Heunis 2001; Heunis and Pringle, 2006). WAA can be a significant phytosanitary issue for fruit exportation and an annoying pest during fruit harvesting (Cockfield and Beers, 2007).

WAA can be controlled chemically by organochloride and organophosphate insecticides (Cockfield and Beers, 2007; Shaw and Wallis, 2009). The international public trend toward the organic farming pushes against the use of chemical control of pests and prompting a need for integrated pest management (IPM). Therefore, adopting biological control through the introduction of the natural endoparasitoid *Aphelinus mali* is a good choice (Brown and Schmitt, 1994; Asante, 1997). However, its susceptibility to many of the insecticides and fungicides commonly used in

apple orchards limits its use (Cohen *et al*., 1996, Nicholas *et al*., 2005; Rogers *et al*., 2011). Cultural control still offers a valuable mean to control WAA in apple orchards through the use of host-plant-resistance (HPR) strategy (Webster, 2003). HPR is an important easy-to-apply tactic of IPM (Beers *et al*., 1993) and can result from morphological factors and activation of defense and signaling responses (Pedigo, 2006). The presence of different apple cultivars and rootstocks provides the opportunity to select the most resistant genotypes available. Variation in susceptibility of apple cultivars to WAA infestations has been previously recorded (Asante *et al*., 1993; Deng *et al*., 1993; Sandanayaka *et al*., 2003). The development of new resistant lines and understanding their genetic background of WAA resistance are significant aspects for foresighted breeding strategies (Brown and Maloney, 2003; Sandanayaka *et al*., 2005). Molecular studies have been conducted for exploring the genetic basis of aphid resistance in apple. Some quantitative trait loci (QTLs) were identified associated with aphid resistance in apple. Molecular markers were discovered linked to QTLs for resistance to the rosy apple aphid, the leaf-curling aphid and the green apple aphid (Stoeckli *et al*., 2008). Recently, progresses have been achieved regarding the development of molecular markers associated with WAA resistance genes (i.e. *Er1*, *Er2* and *Er3*) (Bus *et al*., 2008). Current phenotyping methods for selection of plants resistant to WAA are cumbersome, environment-dependent, and cannot distinguish plants containing combinations of resistance genes from those carrying a single gene (Bus *et al*., 2008). Therefore, screening for the presence of molecular markers linked to WAA resistance genes would be an alternative.

In the current research, different apple cultivars were screened phenotypically for their resistance to WAA infestation. Furthermore, a molecular study was performed for screening these cultivars for the presence of different molecular markers associated with *Er1*, *Er2* and *Er3* resistance genes.

Materials and methods

Monitoring of WAA infestation and data analysis

Monitoring part of the experiment was conducted at the Apple Genetic Complex in Ash-Shoubak Regional Center of Agriculture Research and Extension. The trees under investigations were trained under the central-leader system and were planted on Merton-Malling Series (MM) 106 rootstock. The experiment was conducted on 22 apple cultivars of different flowering time, fruit color, and time of fruit maturity.

WAA infestation was monitored in terms of aerial colonies formation. Monitoring was carried out every 2 weeks on five trees per cultivar for the duration of seven months from January to July. Each tree was rated on a 0-4 infestation rating scale, using a visual indexing technique and ratings modified from Bower (1987). Cultivars were ranked for their relative resistance/susceptibility to WAA based on the tree infestation rating according to Ateyyat and Al-Antary (2009) (Tab. 1).

Mean separation was performed by using Least Significant Differences test (LSD). All differences were compared at the 5 % level ($p \leq 0.05$) of significance and LSD test was used to separate means if significant differences found.

Tab. 1. Rating scale for the assessment of level of resistance based on infestation level of WAA

Level of infestation (%)	Scale	Resistance rating
0	0	Immune
1-15%	0.5	Highly resistant
16-30%	1	Resistant
31-50%	1.5	Moderately resistant
51-65%	2	Moderately susceptible
66-80%	2.5	Susceptible
81-90%	3	Highly susceptible
91-100%	4	Very highly susceptible

Molecular analysis

Healthy leaf tissues were collected from all cultivars under study and were stored at -20 °C for DNA extraction. Leaf tissues were ground into a fine powder in liquid nitrogen using a mortar with a pestle. Template DNA was extracted using a modified CTAB method (Saghi-Maroof *et al*., 1984). For each cultivars, about 0.5 g of fine leaf powder were suspended in 2 ml of DNA extraction buffer (0.22 M EDTA pH 8, 0.8 M NaCl, 0.14 M sorbitol, 0.2 M Tris-HCl pH 8, 0.8% CTAB, 0.5% SDS, 0.5% β-mercaptoethanol). About 500 µl of chloroform were added to the suspension and mixed vigorously. The mixture was incubated at 65 °C for 40 min and then centrifuged. The supernatant was taken and the DNA was precipitated by the addition of 500 µl of isopropanol. The DNA was collected by centrifugation and the DNA pellet was washed twice with 70% ethanol. DNA pellets were dissolved in 100 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA pH 8). The concentration and purity of DNA were assayed using UV spectrometry at 260 and 280 nm.

PCR reactions were performed in purpose of screening the presence of eight different molecular markers linked to major WAA resistance genes (*Er1*, *Er2*, and *Er3*). The molecular markers were amplified using specific primers as suggested by Bus *et al*. (2008) (Tab. 2). All PCR amplification reactions were performed in a total volume of 25 µl. The PCR reaction mixture contained about 300 ng of template DNA, 12.5 µl of 2x PCR master mix solution (iNtRON, Korea), 5 pmole of each primer and distilled H_2O was added to make up the final volume. Markers NZsc_GS327, NZsc_O05, NZsc_E01 and NZsc_A01 were amplified with an annealing temperature of 60 °C and an extension time of 2 min. Whereas, a touchdown PCR cycling regime was employed to amplify the rest of the markers with annealing temperature decreasing from 65 °C to 60 °C in decrements of 0.5 °C per cycle.

PCR amplified products were separated and analyzed by electrophoresis on 1-2% agarose gels stained with ethidium bromide. As molecular weight markers, 1-kp and 100-bp DNA ladders (New England Biolabs) were used. Results were scored for the presence and absence of the molecular markers.

Tab. 2. Primer sequences and product length in base pairs of the molecular markers used in the present study

Marker name	WAA resistance gene	Primer sequence (5'→3')		Product size (bp)
		Forward	Reverse	
NZsc_C20	*Erl*	TCTCTAACTCAATAACTCCCAAGAC	ACTTCGCCACCATTATCACTCCTGA	2,000
NZsc_GS327	*Erl*	GCCAAGCTTCAATGTCGGAGTAGAT	CAAGCTTCCCCTAAGGCTATTGCCA	1,600
NZsc_O05	*Erl*	CCCAGTCACTAACATAATTGGCACA	CCCAGTCACTGGCAAGAGAAATTAC	1,700
NZms_EB145764	*Er2*	TTCCAGCGATCCAAAACAAT	GCTCAGGAACACCTCGTTCT	198
NZsn_O05	*Erl/Er3*	AACGTCATGTCAATAT	CCCAGTCACTGGCAAGAGAAATTAC	880
NZms_EB106753	*Erl/Er3*	TCTGAGGCTCCCAAGTCC	TAGGAGCAGAAGAGGTGACG	175
NZsc_E01	*Er3*	CCCAAGGTCCGAACACAAATGAGAG	CCCAAGGTCCAAAACTATCCCGAAG	1,350
NZsc_A01	*Er3*	CAGGCCCTTCAGCAAAGAGGTGTCT	CAGGCCCTTCACTACTAATAAGAAC	1,250

Results and Discussion

WAA is a potentially detriment pest to apple production worldwide causing a range of diverse injuries to apple trees and fruits (Brown *et al*., 1995). Management of WAA infestations using apple cultivars and rootstocks is considered an important strategy. In the present study, apple cultivars responded differentially to WAA infestation. Results represented in Fig. 1 showed that the highest number of WAA colonies were recorded for five cultivars, namely; 'Fuji *6*', 'Jonathan', 'Red Chief', 'Gold Iralis', and 'Nagava *6*'. Out of the 22 apple cultivars under investigation, cultivars 'Golden Delicious', 'Delbarestivale', 'Golden Smoothie', 'Red Miracle', and 'Harmony' were recorded with complete absence of WAA colonies.

Cultivars ranking based on the tree infestation rating were followed as suggested by Ateyyat and Al-Antary (2009). According to this ranking, 'Fuji *6*' was recorded as highly susceptible cultivar, while 'Golden Delicious', 'Delbarestivale', 'Golden Smoothie', 'Red Miracle' and 'Harmony' were considered as immune (Fig. 2). Ateyyat and Al-Antary (2009) reported that WAA adults failed to colonize on 'Harmony' and no infestations were recorded either on its roots or shoots.

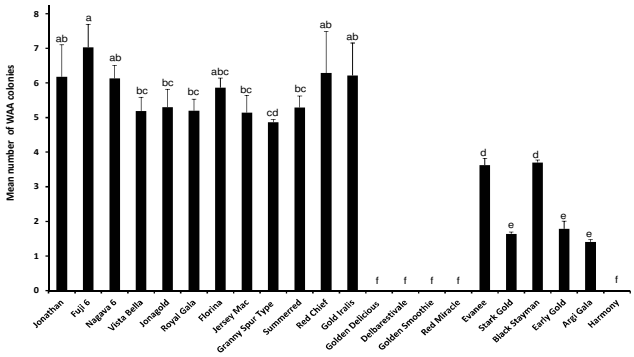

Fig. 1. Mean numbers of WAA colonies per 15 cm length limb of un-sprayed apple cultivars. Means with different letters are significantly different according to LSD

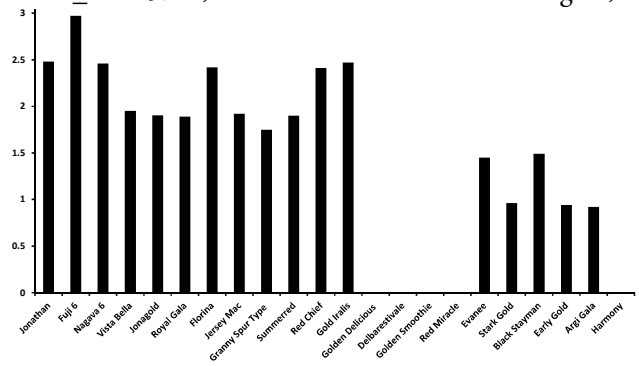

Fig. 2. Shoot infestation rating (0-4) of WAA on un-sprayed apple cultivars

Moreover, the present study yielded three resistant ('Stark Gold', 'Early Gold' and 'Argi Gala'), two moderately resistant ('Evanee', and 'Black Stayman'), six moderately susceptible ('Vista Bella', 'Jonagold', 'Royal Gala', 'Jersey Mac', 'Granny Spur Type' and 'Summerred'), and five susceptible ('Jonathan', 'Nagava *6*', 'Florina', 'Red Chief' and 'Gold Iralis') cultivars. 'Royal Gala' is well-known cultivar for its susceptibility to WAA infestation. This cultivar was reported to display a range of WAA susceptible characteristics including high level of settlement, high survival rate, and long periods of phloem feeding (Sandanayaka *et al*., 2005). None of the 22 apple cultivars tested were ranked as highly resistance or very highly susceptible (Fig. 2). Various WAA-resistant rootstocks were previously developed from the immune cultivars 'northern Spy', including the Malling-Merton rootstocks of the 100 series (Webster, 2003). These rootstocks may owe their resistance to high phenolic levels (Sen Gupta and Miles, 1975). However, some reports have indicated the breakdown of resistance of these rootstocks (Rock and Zeiger, 1974; Ateyyat and Al-Antary, 2009).

Three major WAA resistant genes (*Er1*, *Er2*, and *Er3*) have been characterized and used in apple resistance breeding (Bus *et al*., 2008). Eight different molecular markers linked to these genes were screened by PCR in the apple cultivars under investigation (Tab. 3). The marker NZms_EB145764, which is linked to *Er2* resistant gene, is present in all cultivars. The same is applicable to NZms_EB106753 and NZsc_E01 which are linked to *Er1/Er3* and *Er3*, respectively. On the other hand, NZsn_O05 marker is absent from all cultivars under study. NZsc_A01 (linked to *Er3*) is present in all cultivars except for 'Fuji *6*', 'Jonagold', and 'Granny Spur Type'. Sandanayaka *et al*. (2003) reported that *Er3* resistant gene was carried by the cultivar 'Aotea' and this type of mediated resistance was overcome by WAA, indicating that *Er3*-mediated resistance is distinct from *Er1* and *Er2*.

'Early Gold' is the only cultivar containing NZsc_C20 marker which is linked to *Er1*. The marker NZsc_GS327 is present in 11 cultivars, namely; 'Granny Spur Type',

Tab. 3. Detection of molecular markers linked to WAA resistance genes in different apple cultivars. Plus (+) indicates the presence of a marker

	Markers linked to WAA resistance genes							
Cultivars	NZsc_C20	NZsc_GS327	NZsc_O05	NZms_EB145764	NZsn_O05	NZms_EB106753	NZsc_E01	NZsc_A01
'Jonathan'	-	-	-	+	-	+	+	+
'Fuji 6'	-	-	-	+	-	+	+	+
'Nagava 6'	-	-	-	+	-	+	+	+
'Vista Bella'	-	-	-	+	-	+	+	+
'Jonagold'	-	-	-	+	-	+	+	+
'Royal Gala'	-	-	-	+	-	+	+	+
'Florina'	-	-	-	+	-	+	+	+
'Jersey Mac'	-	-	-	+	-	+	+	+
'Granny Spur Type'	-	+	-	+	-	+	+	-
'Summerred'	-	+	-	+	-	+	+	+
'Red Chief'	-	+	-	+	-	+	+	+
'Gold Iralis'	-	-	+	+	-	+	+	+
'Golden Delicious'	-	+	-	+	-	+	+	+
'Delbarestivale'	-	+	-	+	-	+	+	+
'Golden Smoothie'	-	+	-	+	-	+	+	+
'Red Miracle'	-	+	+	+	-	+	+	+
'Evanee'	-	+	+	+	-	+	+	+
'Stark Gold'	-	+	+	+	-	+	+	+
'Black Stayman'	-	+	+	+	-	+	+	+
'Early Gold'	+	-	+	+	-	+	+	+
'Argi Gala'	-	-	+	+	-	+	+	+
'Harmony'	-	+	+	+	-	+	+	+

'Summerred', 'Red Chief', 'Golden Delicious', 'Delbarestivale', 'Golden Smoothie', 'Red Miracle', 'Harmony', 'Stark Gold', 'Black Stayman', and 'Evanee'. NZsc_O05 marker is linked to *Er1* resistant gene and is present in the following eight cultivars: 'Harmony', 'Red Miracle', 'Evanee', 'Stark Gold', 'Black Stayman', 'Early Gold', 'Argi Gala', and 'Gold Iralis'. 'Northern Spy' and 'Robusta 5' are two WAA resistant genotypes and were reported to carry *Er1* and *Er2* resistant genes, respectively (King *et al*., 1991). These genotypes possessed short duration of phloem ingestion suggesting the presence of resistant factors in their phloem tissues. A previous report indicated that the expression of resistance conditioned by *Er1* increased with tree maturity, while resistance conditioned by *Er2* was high at earlier growth stages (Sandanayaka *et al*., 2003).

Although no clear correlations can be made between the molecular data (marker presence and absence) and the phenotypic results (cultivar ranking), the results of the current study indicated that resistance/susceptibility to WAA infestations varied among apple cultivars. These differences among cultivars can potentially be utilized by commercial apple growers to achieve effective pest control which in turn will reduce input costs and increase profitability.

Further studies are required to understand the mechanisms of responses/defense among different apple cultivars to WAA infestation. Such studies might employ the analysis of traits related to (i) pest-deterrent or repellent secondary metabolites, (ii) defense-related enzymes, and (iii) differential expression of defense genes. These traits may be utilized in the future as marker traits to select for resistance to WAA.

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