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Avermectin Trunk Injections: A Promising Approach for Managing the Walnut Husk Fly (*Rhagoletis completa*)

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Abstract: This study examined the larvicidal effect of trunk-injected abamectin and emamectin benzoate against the walnut husk fly (*Rhagoletis completa* Cresson, 1929). Walnut trees in two locations in two years were injected with the pesticides at different concentrations. For the toxicokinetic studies, the active ingredient content was measured in the leaves, flowers, husks, and kernels, using a UHPLC-MS/MS analytical method. The walnut husk fly infestation rates were between 3 and 70% and 10 and 34% for abamectin and emamectin benzoate, respectively, and were much lower compared to those measured for the control. The active ingredient content in the walnut husk showed a positive correlation with the larvicidal effect. The injections had a measurable but unsatisfactory insecticidal effect in the second year, when the economic threshold was exceeded. Trace amounts of the active ingredients were detected in the flowers. The residue analysis showed a declining concentration trend in the leaves over time. The largest quantities were detected in the leaves (≤439 ng/g of abamectin; ≤19,079 ng/g of emamectin benzoate), with concentrations in the husks of orders of magnitude lower (≤5.86 ng/g; ≤50.19 ng/g). The measurements showed no active ingredient residue above the MRLs in either fresh or dried kernels. The results indicate that trunk injections of abamectin, as well as trunk injections of emamectin benzoate, have the potential to suppress walnut husk fly populations.

Keywords: trunk injection; *Juglans regia*; walnut husk fly; abamectin; emamectin benzoate; tree nuts

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1. Introduction

The English walnut (*Juglans regia* L., 1753) is one of the most widely grown nuts in Europe, with a cultivated area of 154,160 ha and a total yield of 344,728 t/year [1]. Recent years have seen drops in the volumes and average yields of European walnut production despite the establishment of new orchards [1,2], which is in part due to the detrimental effects of climate change and, mainly, the appearance of the walnut husk fly (*Rhagoletis completa* Cresson, 1929). This invasive pest, which is native to central and eastern America and northeast Mexico, was first recorded in Europe in Switzerland [3–5] and was first identified in orchards in 1991 in Italy [6], from where it spread to France, Spain, Germany, Austria, Croatia, Slovenia, and Hungary [7]. As the species has not yet reached the ecologically delimited boundaries of its distribution, it is expected to arrive in all walnut-growing areas of Europe and Asia [7,8].

In Europe, its primary threat is to the English walnut (*Juglans regia*); however, there are significant differences among cultivars in susceptibility [9–11]. In the Carpathian Basin,

most adults tend to fly from the beginning of July until the end of August. The exocarp shows a small, inconspicuous spot of discoloration where the egg is laid. As the maggots grow, irregularly shaped black spots of increasingly large dimensions appear on the outside of the walnut [12,13]. As a result of the damage, dark spots can also be observed on the endocarp [14], the kernel can become wrinkled, pitted, and rotten, and, secondary, saprophytic pathogens can also appear on its surface [10]. The damaged walnuts can be expected to drop before becoming fully ripe, and their weight, oil, and protein content also suffer greatly [15]. An even greater problem for producers is that the damage negatively affects marketability indicators (darker kernel, spotty shell). The post-harvest treatment of the yield is made more expensive by the decaying or dried black husk that sticks to the shell, as its removal increases the cleaning costs [16,17].

Protection against this pest is a difficult task, limited by the walnut husk fly's (WHF) long period of emergence and the physical characteristics of walnut orchards [18,19]. The orchards require continuous protection during the emergence of the WHF, which consists of several treatments, usually involving aerial spraying and baiting [20–22].

One non-spray method that has been studied for controlling WHF is the trunk injection of the insecticides abamectin (ABA) and emamectin benzoate (EMA). Although the history of trunk injection stretches back to the 12th century, the first experiments date back only to the beginning of the 20th century [23–25]. Climate change has contributed in large part to the increase in the use of injection solutions due to the prevalence of invasive species [26]. Several methods can be used for trunk injection [27,28], but they are similar in that they generally target the tree's water transport system (xylem), where the introduced active ingredient (AI) reaches the site of action with the help of the forces of transpiration [26,29]. This procedure has numerous advantages over traditional spraying methods [30]. One of its biggest advantages is its more acceptable ecotoxicological profile (no wash-off, no drift, the AI completely reaches the target location, no/less water is required for its application) [28]. Due to the lower AI exposure to workers achieved with this method, human toxicology indicators are also better than for spraying [31].

A number of studies have reported on the successful use of trunk injection, regardless of whether the purpose was to stop pathogen microorganisms [32–35] or insect pests [36,37]. ABA endotherapy was used successfully when canker disease of the walnut emerged, caused by the pathogen *Geosmithia morbida* Kolařik, 2010, and vectored by the bark beetle (*Pityophthorus juglandis* Blackman, 1928) [38]. EMA injection in ashes showed high efficiency against *Agrilus planipennis* Fairmaire, 1888, of the ash for several years [39,40].

Avermectins are derivatives of natural compounds belonging to the class of macrocyclic lactones that have nematicidal, acaricidal, and insecticidal effects [41]. In spray applications, ABA is commonly used against pests with piercing–sucking mouth organs and against leaf miner larvae, while EMA is mainly used against fruit and leaf miner moths [42–44]. The semi-synthetic EMA has improved thermal stability and greater water solubility than ABA, which can make it more suitable for trunk injection purposes [45].

The physicochemical parameters of ABA and EMA make them suitable for injection, even though they are not labelled against the walnut husk fly. However, the biological spectrum of a given active substance depends not only on whether it is able to exert biological effects on organisms (toxicodynamic factor) but also on whether the pest comes in contact with the compound that would otherwise be effective (toxicokinetic factor) [46]. Products containing these AIs are also commercially used for injecting horse chestnut (*Aesculus hippocastanum* L., 1753) against its leaf miner moth (*Cameraria ochridella* Deschka & Dimic, 1986) [47]. To the best of our knowledge, only one experiment reporting the use of ABA against the WHF has been published so far, in which the injected ABA successfully controlled the WHF [48]. However, that study was preliminary with inconclusive results and did not measure the insecticide levels in different parts of the tree.

The objective of this study was to examine the larvicidal effect of trunk-injected ABA and EMA on the WHF and to evaluate the resulting economic damage. We also evaluated the toxicokinetic behavior of these active substances applied via endotherapy through the

determination of chemical residues. Our research stands out for its comprehensive analysis of the temporal residue profiles of compounds within the canopy, as well as of their efficacy one year after the injection. Furthermore, we identified some potential side effects that should be taken into consideration.

2. Materials and Methods

2.1. Experimental Sites

The trunk injections were performed in two years at two locations 100 km from each other: in 2020, in Taksony (Trial I.), Hungary (GPS: 47.306584, 19.083406); in 2021, in Szelevény (Trial II.), Hungary (GPS: 46.827162, 20.170184). Both sites have a temperate continental climate; the annual average mean temperature is 12 °C, and the rainfall is 550 mm. The size of the plantations was 0.5 and 1 ha, respectively. No other plant protection products were applied to either the trees involved in the experiment or any trees in the orchard. Both experiments were performed on the variety Alsószentiváni 117 of the English walnut. The planting density was 10 × 10 m at both sites. At the outset of the experiment, the trees were 11 years old in Trial I, with an average height of 5 m, and 35 years old in Trial II, with an average height of 8 m; accordingly, the trunk diameters were between 16 and 20 cm and between 28 and 34 cm.

2.2. Injection Method

The experimental design was completely randomized. Three replicates were set up for each treatment. For the study, a total of 45 trees were used, and two types of controls were designated: (1) a water-based control (C aq.), with the injection of only water, and (2) no injection (C no inj.).

Two different tools with similar principles were used for injecting. Both tools directly were connected to the hole previously drilled for the injection. The injection points were 20 cm above the ground level. After the injections, the wounds were closed with a tree gel (FAGÉL, FÉNYLAKK Kft.).

In the case of the tool Treenject (Figure S1), 4–8 (depending on the trunk diameter) 50 mm deep holes with a 3.5 mm diameter were drilled around the trunks at equal distances using an electric drill and a clean, sharp bit. This tool can inject a small (10–20 mL/tree) amount of liquid with a maximum pressure of 12.6 bar. The other tool (Figure S2) was a combination of a pressurized rubber bag and an applicator pipe (Ynject GO, Fertinyect S.L., Córdoba, Spain). The application with the second tool involved four 6.5 mm diameter drilled holes with a depth of 50 mm around the trunk. Using this tool, we could inject a high (60–200 mL/tree) amount of liquid.

The pesticide products containing 18 g/L of ABA (Vertimec 1.8 EC, Syngenta, Basel, Switzerland) and 95 g/L of EMA (Revive II, Syngenta) were used separately, as they are readily available in the Hungarian market. The formulation of the first product was developed for foliar application, while the second one was specially designed for trunk injection. The treatments took place during the trees' intensive growth phase, approximately 4–6 weeks before the expected emergence of adults (Table 1). The injections were performed between 10 a.m. and 3 p.m. on sunny days.

2.3. Sampling

The plant samples were randomly collected from all parts of the tree canopy to examine insecticidal effect and pesticide residues. When taking the samples, 60 compound leaves were collected from each tree replicate (with the tree being divided into four parts according to the four cardinal points) at the times indicated in Table 1, as was 500 g of flowers (for the chemical analysis, we divided it into three parallel samples) from each tree in full bloom. During the fruit sampling, 100–150 husked walnuts were collected in Raschel bags from each tree. After evaluating the insecticidal effect (Section 2.4), three parallel samples of 500 g of husk were made from the collected fruits to determine the active ingredient content

for each tree. To avoid the unwanted wetting of the samples, collection was performed on sunny days.

Table 1. Detailed information about the injections and samplings.

Location and Date of Injection	AI	Injection Parameters			Injection Method	Days after Treatment (DAT)			Numbering of the Trees
		Product (mL/Tree)	Volume (Diluted) (mL/Tree)	AI (g/Tree)		Leaf	Husk and Kernel	Flower	
Taksony 4 June 2020 (Trial I.)	ABA	10	20	0.180	T	57; 108; 138	108; 483	348	1; 2;3
	ABA	20	20	0.360	T	57; 108; 138; 348; 463; 483			4; 5; 6
	EMA	5	10	0.475	T				7; 8; 9
	EMA	10	10	0.950	T				10; 11; 12
	C aq.	-	10	-	T				13; 14; 15
	C no inj.	-	-	-	-				16; 17; 18
Szelevény 28 May 2021 (Trial II.)	ABA	50	100	0.900	Y	35; 71; 106	111	337	19; 20; 21
	ABA	100	100	1.800	T				22; 23; 24
	ABA	100	200	1.800	Y				25; 26; 27
	ABA	200	200	3.600	Y				28; 29; 30
	EMA	20	60	1.900	Y				31; 32; 33
	EMA	40	60	3.800	Y				34; 35; 36
	EMA	60	60	5.700	Y				37; 38; 39
	C aq.	-	100	-	Y				40; 41; 42
	C no inj.	-	-	-	-				43; 44; 45

Abbreviations: ABA = abamectin (18 g/L); EMA = emamectin benzoate (95 g/L); AI = active ingredient; C aq. = injected with distilled water; C no inj. = no injection was performed; T = Treenject, Y = Ynject GO.

The samples collected for the residual analyses were kept at −80 °C until analysis. Half of the walnut fruit samples were also stored unfrozen, simulating the traditional postharvest technology (storing at room temperature in a well-ventilated room, spread out), for 4 weeks following harvesting.

2.4. Insecticidal Effect and Rating of the Damage

The insecticidal effect was evaluated based on the presence of live larvae in the walnut husk. The investigation was performed in September, when the husks split but before they dropped from their shells. The husks were examined within 1–2 days of collection by cutting them into eight slices. The fruits were classified into two groups based on whether the husk contained live larvae or not, to determine the infestation rate. The infestation rate was determined as the percentage of total examined nuts (based on 100–150 walnuts per tree replicate) that were damaged, indicating the presence or absence of larvae. Although this variable is a good indicator of the direct insecticidal effect, it is not equivalent to the degree of economic damage, which is more important to walnut production. In light of this fact, a rating to express the extent of economic damage was added to the evaluation in 2021.

The extent of economic damage was estimated based on the frequency of fruits displaying black spots and the occurrence of such spotting on each nut. Of these two parameters, the effect of the treatment on economic or production quality was classified in the group corresponding to the higher rating. The trees were classified into six groups according to the degree of damage (Table 2).

Table 2. Characterization of the groups estimating the economic damage.

Frequency of Spotty Fruit	0	1–10%	11–50%	50–74%	75–99%	100%
The extent of the largest spot visible on each nut, in the percentage of the husk	0	1–10%	11–50%	50–74%	75–89%	>90%
Rating (damage)	0	1	2	3	4	5
Economically acceptable (Yes/No)	Y	Y	Y	N	N	N

2.5. Short- and Long-Term Efficacy of the Endotherapy

Residue determination was carried out over both short- and long-term time periods to evaluate the pesticides' toxicokinetic behavior. For the short-term study, the residue content was measured in leaf samples collected three times during one vegetative period (35, 71, and 106 DAT, Trial II., Table 1), then the residue content was determined. The residue content was also measured in husks and kernels (108 DAT, Trial I., and 111 DAT, Trial II., Table 1).

The long-term efficacy study was performed also in the first year and in the second year following the winter dormancy period. The pesticide residues in the leaf samples were measured over a longer period of time (57, 108, 138, 348, 463, and 483 DAT, Trial I.). The residues were measured in husks (483 DAT, Trial I.), kernels (483 DAT, Trial I.), and flowers (348 DAT, Trial I., 337 DAT, Trial II.) in the second year (Table 1). For evaluating the second-year insecticidal effect, the infestation rates were also examined in the same manner described above (483 DAT, Trial I., Table 1).

2.6. Chemical Analysis

The pesticide residues in the samples were extracted in accordance with EN 15662:2018 [49] using a citrate-buffered QuEChERS sample preparation method. The chemicals used for the residue measurement are described in Kmellár et al. (2010) [50]. In the case of the kernel samples, a defatting step was also integrated into the procedure, which included freezing out and dSPE cleaning with a C18 sorbent. The method involved an extraction with acetonitrile, which facilitates the determination of pesticide residues using a UHPLC-MS/MS-linked technology. For determining the ABA content, an SPE cleaning procedure was also applied after the QuEChERS extraction.

Regarding the instrumental parameters, the Single Residue Method of the AI published by the Community Reference Laboratories for Residues of Pesticides was applied [51,52]. The method validation was performed for the detection limit (DL), quantification limit (QL), extraction efficiency, linearity, and matrix effect. The method validation was performed in accordance with the SANTE guidelines [53]. The DL values in the cases of husk, flower, leaf, and kernel were different (ABA: 1.2 ng/g; 2.4 ng/g; 2.4 ng/g; 2.4 ng/g; 2.4 ng/g; EMA: 0.1 ng/g; 0.2 ng/g; 0.2 ng/g; 0.2 ng/g; 0.2 ng/g, respectively). The calibration curves were linear up to a concentration of 1000 ng/mL of ABA and 1880 ng/mL of EMA, and the matrix effect calculated with the use of the Matuszewski equation [54] was between 61% and 95% and between 70% and 120%, respectively.

2.7. Statistical Analysis

The statistical analyses were performed using IBM SPSS Statistics 27 and Excel 2016 software. As the conditions of the ANOVA were not met for the AI concentrations, a Kruskal–Wallis test was performed to compare the ABA and EMA concentrations. The Marascuilo [55] procedure was used to compare the treatments based on the infestation rates of the trees. The infestation rate (percentage of fruits with live larvae) was calculated for each treatment and presented as total infestation across all replicates. Spearman's rank correlation was used to examine the associations between the variables (injected quantity of AI, infestation rate, residual content). The results were considered significant if $p < 0.05$.

3. Results

3.1. Insecticidal Effect

The infestation in the fruits of the treated trees was significantly lower in the year of injection than in the control group (Figure 1). The husk showed the earliest evidence of the larvicidal effect, and a microscopic examination of the oviposition sites indicated that the 1–2 mm blackish patches were the remains of larvae that had died very early in development beneath the epidermis (Figures S3 and S4).

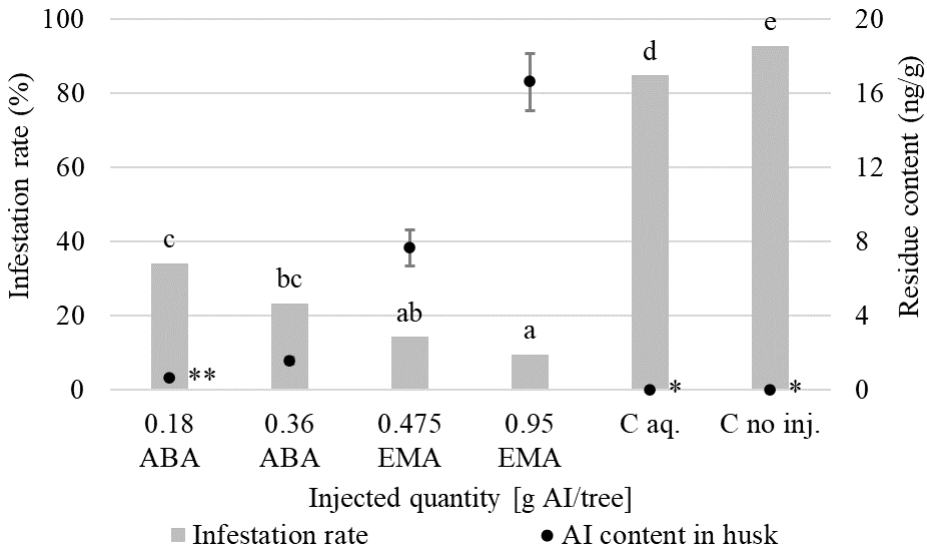

Figure 1. Infestation rate and husk pesticide residue content (mean ± SE). Trial I. For treatments in columns marked with the same letter, the Marascuilo comparison shows that the infestation rate is not significantly different ($p > 0.05$). Location: Taksony, injection date: 4 June 2020, sampling date: 20 September 2020 (108 DAT); detection limit (DL): 1.2 ng/g ABA, 0.1 ng/g EMA. AI = active ingredient; ABA = abamectin; EMA = emamectin benzoate. * <DL, ** trace.

On the trees treated with EMA, the percentages of walnut husks containing live larvae was lower (14% and 9%, respectively) than on trees treated with ABA (34% and 23%, respectively). In the case of both AIs, the higher dose resulted in a better insecticidal effect, although the dose–response relationship was not unequivocally significant. The Marascuilo comparison showed that the higher EMA dose had a significantly better larvicidal effect compared to both doses of ABA. Both the higher dose of ABA and the lower dose of EMA had intermediate effects. Trees injected with water and untreated trees experienced high infestation rates, with husks containing live larvae ranging between 84% and 94%. On trees injected with EMA, the AI content in the walnut husk was 7.65 ± 0.96 ng/g (mean ± SE) for the lower dose and 16.61 ± 1.55 ng/g for the higher dose. In the control, the AI content was below the detection limit (Figure 1).

The percentage of husks with live maggots from the trees treated in 2021 (Trial II.) was significantly lower than in the case of tree subjected to the control treatments. The infestation rate was the lowest on the trees treated with EMA (22% and 34%), with ABA showing slightly worse results (42% and 70%), while the control trees were 100% infested (Figures 2 and 3). The lowest dose of ABA resulted in the weakest insecticidal effect; however, a significantly better effect was found with increased doses of ABA (Figure 2). Accordingly, not only were the infestation rates lower when applying EMA rather than of ABA, but the actual difference in the infestation rates was also lower depending on the injection of the various quantities of AIs. On trees injected with EMA, no difference was observed in the insecticidal effect between the lowest and the highest doses (Figure 3).

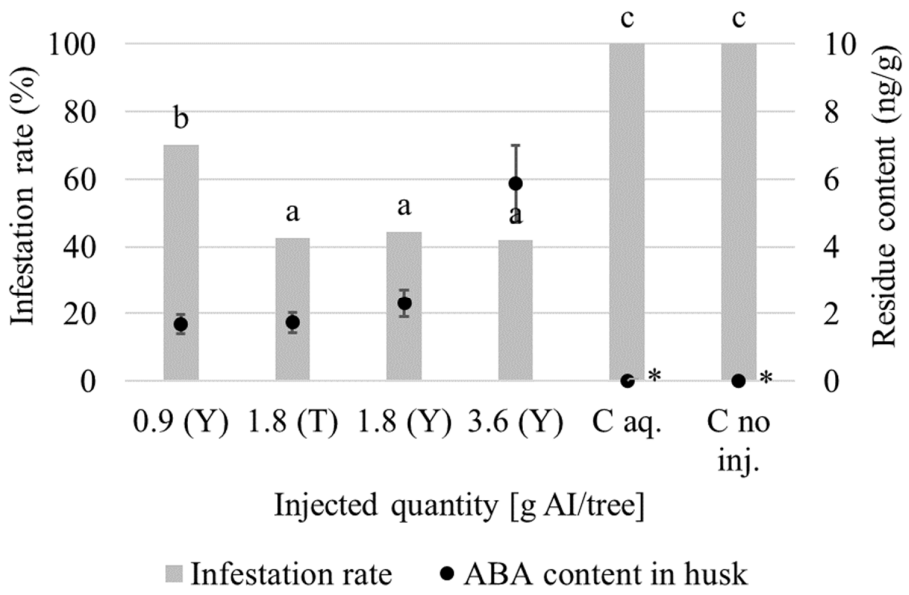

Figure 2. Infestation rate and abamectin content in the husk (mean ± SE) as a consequence of the injection treatments using various tools (T = Treenject, Y = Ynject GO) and doses. Trial II. For treatments in columns marked with the same letter, the Marascuilo comparison shows that the infestation rate is not significantly different ($p > 0.05$). Location: Szelevény, injection date: 28 May 2021, sampling date: 16 September 2021 (111 DAT); detection limit (DL): 1.2 ng/g. AI = active ingredient; ABA = abamectin. * <DL.

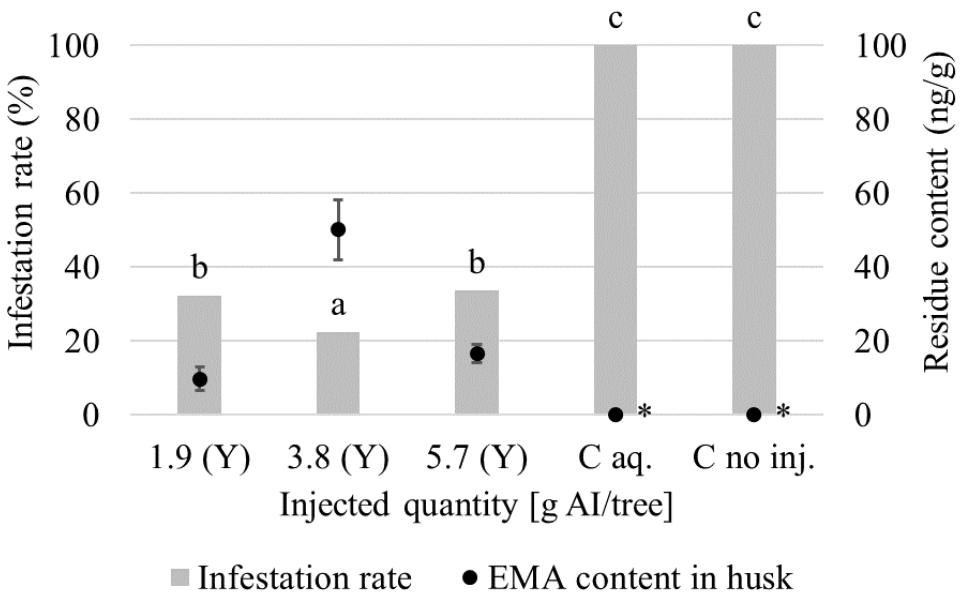

Figure 3. Infestation rate and emamectin benzoate content (mean ± SE) in the husk as a consequence of the injection treatments using various doses (Y = Ynject GO). Trial II. For treatments in columns marked with the same letter, the Marascuilo comparison shows that the infestation rate is not significantly different ($p > 0.05$). Location: Szelevény, injection date: 28 May 2021, sampling date: 16 September 2021 (111 DAT). Detection limit (DL): 0.1 ng/g. AI = active ingredient; EMA = emamectin benzoate. * <DL.

The ABA residue measured in the walnut pericarp showed a slightly positive correlation with the quantity of the injected AI ($r_s = 0.478$) and a negative correlation with the degree of infestation ($r_s = -0.455$), though, statistically, these values did not show a significant relationship ($p = 0.116$ and $p = 0.138$, respectively). The average ABA content

in the husk was between 1.67 ± 0.28 and 5.86 ± 1.14 ng/g, though these seemingly different values did not differ significantly (Kruskal–Wallis H = 3.00, df = 3, p = 0.392).

In the case of EMA, a negative correlation ($r_s = -0.867, p = 0.002$) was identified between the residue content and the infestation rate, and there was no significant correlation between the injected and the residual quantity of AI ($r_s = 0.316, p = 0.407$) (Figure 3). The average content was between 9.69 ± 3.23 and 50.19 ± 8.14 ng/g, but the differences were not significant in this case either (Kruskal–Wallis H = 5.067, df = 2, p = 0.079).

In the 2021 economic damage assessment of the fruits, all trees treated with EMA, and the trees treated with the highest dose of ABA reached an acceptable damage score of 2 or less. The medium dose of ABA was partly acceptable, and its smallest dose fell into the unacceptable economic damage category (Table 3).

Table 3. The assessed economic damage of fruits (husk + kernel) Trial II.

AI Type (Injection Tool)	Injected AI (g/Tree)	Average Rating (0–5)
ABA(Y)	0.9	2.7
ABA(T)	1.8	2.3
ABA(Y)	1.8	2.0
ABA(Y)	3.6	2.0
EMA(Y)	1.9	1.0
EMA(Y)	3.8	0.7
EMA(Y)	5.7	1.0
C aq.	-	5.0
C no inj.	-	5.0

Abbreviations: AI = active ingredient; ABA = abamectin; EMA = emamectin benzoate; T = Treenject tool; Y = Ynject GO tool.

Using two kinds of injection tools (at 1.8 g AI/tree), a significant difference was not identified either between the infestation rates or between the residue content of the husk in the case of ABA (Figure 2). However, in the leaves, the use of the Ynject GO tool resulted in more-than-twice higher concentrations of residue at all three sampling times, compared to that of the Treenject tool (Figure 4a).

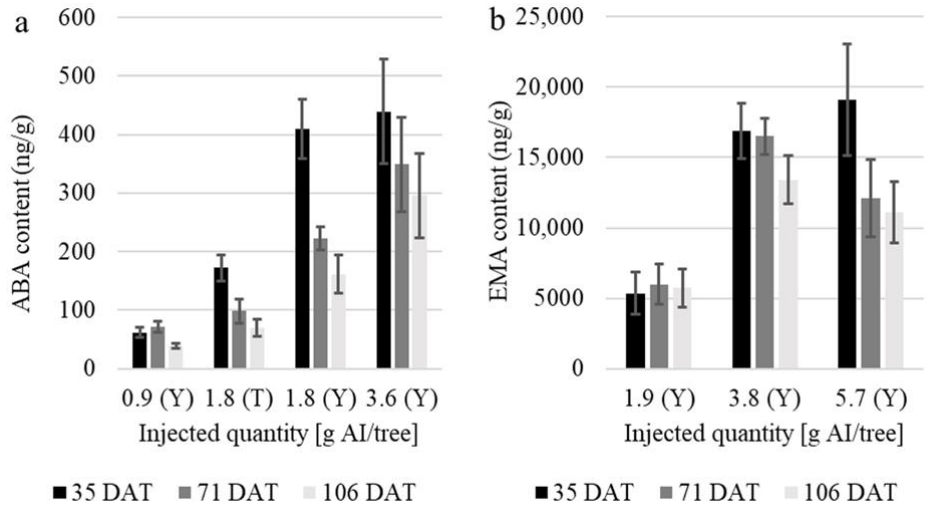

Figure 4. Comparison of the active ingredient content of the leaves (mean ± SE) after abamectin (**a**) and emamectin benzoate (**b**) trunk injections at 3 different times and comparing two different tools (T = Treenject, Y = Ynject GO) (**a**). Trial II. Location: Szelevény, injection date: 28 May 2021; detection limit: 2.4 ng/g ABA, 0.2 ng/g EMA. AI = active ingredient; ABA = abamectin; EMA = emamectin benzoate; DAT = days after treatment.

3.2. Short-Term Residue Monitoring in the Leaves

In general, the pesticide concentration determined in the leaf samples gradually declined over the sampling period. The smallest injection quantity was an exception, as the active ingredient content of the samples collected on the 71st day was slightly higher for both AIs than in the samples collected on the 35th day (Figure 4). The pesticide residue content in colored leaves taken during the autumnal leaf drop was lower than in green leaves collected at the same time, which were mostly still assimilating (Figure 5).

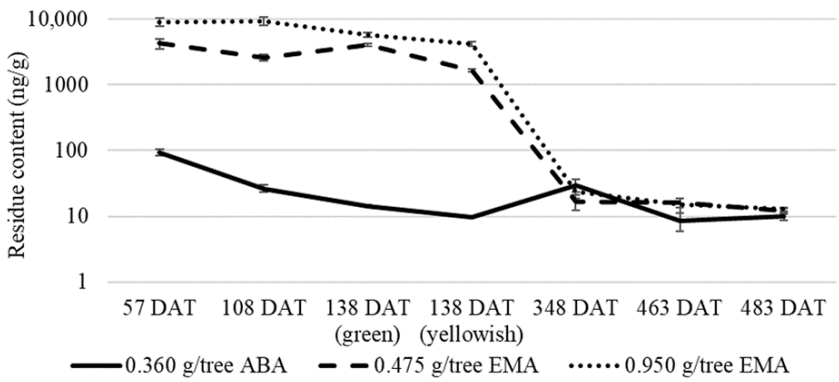

Figure 5. Residue content of the leaf samples collected throughout 2 years (2020–2021) following the injection (with the Treenject tool). Trial I. ABA = abamectin; EMA = emamectin benzoate; DAT = days after treatment.

During the short-term monitoring, a positive correlation between the injected and the residual AI content was shown in the leaves (Figure 4a), with $r_s = 0.819 (p = 0.001)$ on the 35th day, $r_s = 0.785 (p = 0.002)$ on the 71st day, and $r_s = 0.853 (p < 0.001)$ on the 106th day. The positive correlation was weaker but significant between the injected and the residual EMA content in the leaves on the 35th day, with $r_s = 0.685 (p = 0.042)$, and was not significant on the 71st and 106th days ($r_s = 0.422, p = 0.258$ and $r_s = 0.580$, $p = 0.102$, respectively). According to Figure 4b, for reasons unknown, the seemingly logical connection between the injected and the mean residual EMA content no longer existed on the 71st and 106th days at the two higher concentrations.

3.3. Long-Term Efficacy of the Endotherapy

Leaf samples were collected throughout two vegetation periods, between 57 and 483 DAT (Trial I., Table 1). Figure 5 shows that both AIs appeared for at least two years, though the quantity in the second year was orders of magnitude lower than in the first year.

In terms of a long-term effect, the most important question focuses on the residue content in the husk, where the larvae develop. Biological efficacy as well as residue content were investigated in the husk samples collected in the second year of injection (483 DAT). Although these trees still displayed a detectable insecticidal effect, it was much lower than in the first year: the infestation rate was 65% in ABA-injected trees and 60% in EMA-injected trees, with the controls showing a 91–92% infestation rate (Figure 6).

3.4. Residue in the Flowers

The ABA content of the flower samples collected from trees injected the previous year was below the detection limit, and only a trace of EMA could be detected (Table 4).

3.5. Residue in the Kernels

The ABA residual content in the kernels did not exceed the detection limit (2.4 ng/g) in either the short-term or the long-term monitoring, while the EMA content stayed below

the detection limit (0.2 ng/g), with one exception, where it amounted to 0.5 ng/g (tree No. 11., DAT 108).

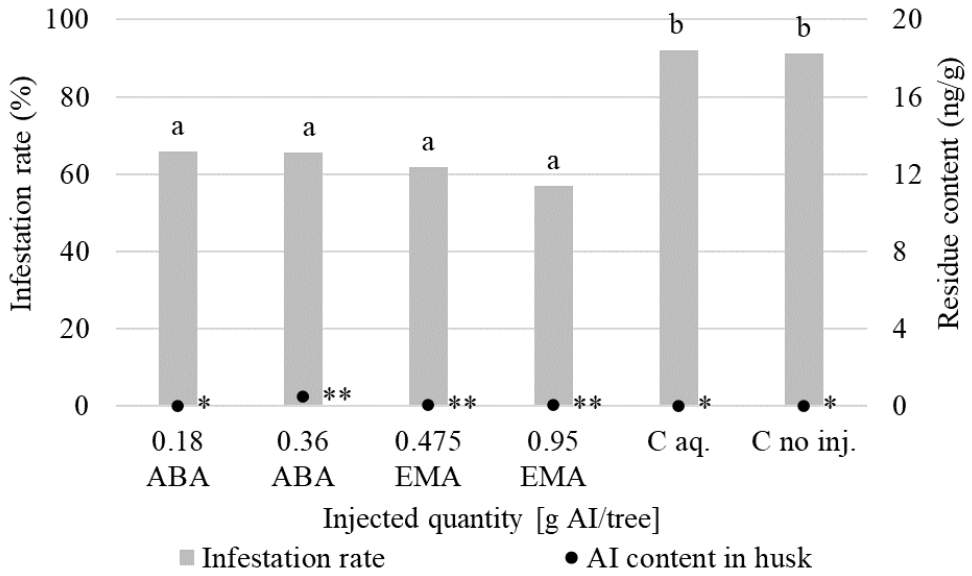

Figure 6. Infestation rate and residue content in the husks (mean ± SE) in the second year following the trunk injections. Trial I. For treatments in columns marked with the same letter, the Marascuilo comparison shows that the infestation rate is not significantly different ($p > 0.05$). Location: Taksony, injection date: 4 June 2020, sampling date: 30 September 2021 (483 DAT); detection limit: 1.2 ng/g ABA, 0.1 ng/g EMA. AI = active ingredient; C aq. = injected with distilled water; C no inj. = no injection was performed; ABA = abamectin; EMA = emamectin benzoate. * <DL, ** trace.

Table 4. Residual content of the flowers in the next spring after the injection (Taksony, Hungary, 2021). Trial I.

Trial	Treatment (Tool)	Amount of Injected Active Ingredient (g/Tree)	Pesticide Residue in 2nd-Year Flowers (337–348 DAT) Min-Max (ng/g)
Trial I.	ABA (T)	0.180	<DL
	ABA (T)	0.360	<DL
	EMA (T)	0.475	1.46–2.31
	EMA (T)	0.950	1.84–2.01
	Caq. (T)	-	<DL
	C no inj.	-	<DL
Trial II.	ABA (Y)	0.9	<DL
	ABA (T)	1.8	<DL
	ABA (Y)	1.8	<DL
	ABA (Y)	3.6	<DL
	EMA (Y)	1.9	0.39–0.73
	EMA (Y)	3.8	0.64–1.46
	EMA (Y)	5.7	0.28–1.63
	C aq. (Y)	-	<DL
	C no inj.	-	<DL

Trial I.: Injection on 4 June 2020; samples collected on 18 May 2021 (348 DAT). Trial II.: Injection on 28 May 2021; samples collected on 30 April 2022 (337 DAT). DL_{ABA}: 2.4 ng/g; DL_{EMA}: 0.2 ng/g. Abbreviations: ABA = abamectin; EMA = emamectin benzoate; DL = detection limit; DAT = days after treatment; T = Treenject; Y = Ynject GO.

4. Discussion

Trunk injection led to the appearance of both AIs in green plant parts, including leaves and husks, and EMA was also detectable in the flowers. The concentration of EMA

in the plant parts was higher than that of ABA due to the presence of basic nitrogen, which increased its polarity and caused it to become protonated, leading to a greater water solubility [45].

On the injected trees, eggs were laid in the same manner and number as on the control trees. No repellent effect was observed, and it is assumed that the AIs did not exert an ovicidal effect on eggs laid in the husk. They did kill the larvae after they emerged from the eggs and started to feed. These dead, shriveled maggots and the eggshells were found under the exocarp. The oviposition was easily distinguished on the husk, identified by small, black, dry spots, though these did not cause any reduction in quality and any secondary infestation in the kernel or had any negative effect on the success of walnut production (Figure S5). Trunk injection can be used to prevent larval damage; it cannot be used to kill adults or prevent egg laying.

The infestation rates were significantly different between the two sites for both pesticides (Figures 1–3). The authors believe that this was due to the difference in distribution of the pesticide in the two years, as transpiration is greatly influenced by the soil water content [25,28,56], and to the increased presence of the pest. When considering these data, the strictness of the evaluation method must be taken into consideration, as the infestation of the husk does not necessarily mean that the kernel is unsaleable. If any surviving larvae remain in the husk, the AIs can still exert their sublethal effects, slowing maggot development. The husk may turn black in part, but the economic damage might be low, as the kernel may retain its marketability. Our data about the economic damage related to the produced walnut showed that the high dose of ABA and all EMA treatments provided a sufficient insecticidal effect (Table 2).

In the case of the EMA treatment, in contrast to the ABA treatment, the infestation rate did not consistently correspond to the dose applied. However, there was a negative correlation between the residue content of the husks and the infestation rate. This can be a result of the fact that the trunk diameters and the canopies of the trees were not always directly proportional, though the manufacturers clearly recommend determining the dose based on the trunk diameter at breast height (DBH) [57,58].

The higher infestation in the second year indicated that the treatment must be repeated annually in the case of the examined pesticides. Although the difference in the infestation rates was significant between the treated and the control trees, the resulting drop of the biological effect in the second year showed that the treatments were no longer effective for practical purposes. This can be explained by the low pesticide residues in the pericarp, which also showed a sharp drop compared to the first year (Figures 1 and 6). Several studies reported the long-lasting efficiency of trunk injection [33,34,37,59], but the differences between tree species, pests, and pesticides perhaps make it impossible to draw comparisons based solely on the literature [58,60]. The decrease in effectiveness seen in the second year is consistent with the decreasing concentration of pesticide residues in the leaves over time (Figure 5).

In most cases, the concentration of the active substances in the leaves decreased over the course of the sampling, except for the smallest treatment concentration (0.9, ABA; 1.9, EMA), where a slightly higher value was measured on the 71st day than on the 35th day (Figure 4). This phenomenon can be explained by the fact that the smaller concentration took a longer time to reach the canopy, meaning the maximum concentration in the canopy was achieved somewhere between the 35th and the 71st day.

One disadvantage of the injection may be the effects that wounds on the trunk can exert on the performance and health of the wood. Regardless of the dosage, the trunks treated with the foliar spray formulation (Vertimec 1.8 EC, Figure S6) displayed extensive phytotoxicity, while no phytotoxicity was observed resulting from the use of the plant protection product developed specifically for trunk injection (Revive II, Figure S7). Although the phytotoxic symptoms disappeared in the second year following the treatment, the wound closure was not ideal, contrary to what observed for the product intended for trunk

injection. It is strongly recommended to use products formulated specifically for trunk injection without any additives that may have detrimental effects on the trunk tissue.

Due to the risks to the health of the trunk [32,61] and the necessity of repeating the treatment every year, it is recommended to develop a non-invasive method that requires no drilling. This could be a form of needle-based injection [28] or a basal bark spray treatment with a special adjuvant system [62–66]. According to our assumption, planting a stainless valve in the trunk could also serve this purpose, eliminating the need to create holes every year.

The concentration of pesticide residues in the kernel did not exceed the MRL (Maximum Residue Limit) values specified in the EU pesticide database (ABA: 0.02 mg/kg; EMA: 0.01 mg/kg), meaning that the trunk injection was suitable from the perspective of food safety.

The authors believe that trunk injection is fundamentally more eco-friendly than foliar sprays. Although it offers a number of advantages [67,68], certain risks were also identified (wound healing, the role of the formula, the uniform and sufficient translocation of the AIs) that appear to be avoidable with the introduction of additional developments [69–71]. The study found that the flowers from the injected walnut trees did not contain any detectable level of ABA residue, and trace amounts of EMA residue were present in the flowers, similar to the levels found in apple nectar and pollen by Coslor et al. (2019) [72]. This is a promising result, as it suggests that the injections had a minimal impact on the presence of pesticide residues in the flowers. However, the difficulties in assessing the pesticides' impact on flower-visiting insects require complex tests in the future [73,74].

This alternative method can be used to achieve results analogous to those obtained with traditional foliar sprays, where 4–5 foliar spray treatments are usually required to ensure coverage throughout the emergence period. In terms of costs, it is similar to traditional spraying on an annual basis. In Hungary, walnut production is based both on individual orchards and on backyard-kept walnut trees. Protecting walnuts grown within urban areas is limited or not realistic, which is why the authors consider trunk injection to be an alternative solution suitable for these areas.

Trunk injection in walnut against the WHF has probably also a beneficial insecticidal effect against other substantial pests such as the codling moth (*Cydia pomonella* L., 1758), which should also be examined in the future.

5. Conclusions

Our research provides insights into how the trunk injection technology can protect plants from the WHF, walnut's most important pest. The application of both ABA and EMA via trunk injection was found to be successful in preventing damage caused by the WHF, but only in the year of the injection and not in the following year.

The residues measured in either fresh or dried kernels did not exceed the maximum residue limit, which indicates the safety of the tested compounds applied via endotherapy for walnut pest control.

Our study highlights the potential of the trunk injection technology as a viable option for the management of the WHF, providing practical guidance.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae9060655/s1. Figure S1: Treenject injection tool; Figure S2: Pressurised rubber injection bag (Ynject GO); Figure S3: The oviposition site on the green walnut husk (left side) and the dead young maggots in the mesocarp (right side); Figure S4: The live maggots in the pericarp (right side) and the maggots killed as a result of the treatment (left side); Figure S5: Small, black, dry spots on the husk as a consequence of oviposition; Figure S6: Side effects due to the use of a plant protection product not suited for injection in the year of the treatment (left, 15 October 2020) and one year later (right, 18 May 2021); Figure S7: Absence of side effects due to the use of a plant protection product intended for trunk injection in the year of the treatment (left, 15 October 2020) and one year later (right, 18 May 2021).

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