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Residue dynamics of a contact and a systemic fungicide in pollen, nectar, and other plant matrices of courgette (Cucurbita pepo L.)

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Residue dynamics of a contact and a systemic fungicide in pollen, nectar, and other plant matrices of courgette (*Cucurbita pepo* L.)☆

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ABSTRACT

Pollen and nectar can be contaminated with a range of pesticides, including insecticides, fungicides, and herbicides. Since these matrices are important food sources for pollinators and other beneficial insects, their contamination can represent a key route of exposure. However, limited knowledge exists with respect to pesticide residue levels and their dynamics in these matrices for many crops and active ingredients (AIs). We used controlled glasshouse studies to investigate the residue dynamics of a systemic (cyprodinil) and a contact (fludioxonil) fungicide in the floral matrices and other plant parts of courgette/zucchini (*Cucurbita pepo* L.). We aimed to better understand the processes behind residue accumulation and decline in pollen and nectar. Each AI was applied to plants, either by spraying whole plants or by targeted spraying onto leaves only. Samples of pollen, nectar, anthers, flowers, and leaves were taken on the day of application and each subsequent morning for up to 13 days and analysed for residues using LC-MS/MS. Significant differences in residue levels and dynamics were found between AIs and floral matrices. The present study allowed for the identification of potential routes by which residues translocate between tissues and to link those to the physicochemical properties of each AI, which may facilitate the prediction of residue levels in pollen and nectar. Residues of the contact AI declined more quickly than those of the systemic AI in pollen and nectar. Our results further suggest that the risk of oral exposure for pollinators may be considerably reduced by using contact AIs during the green bud stage of plants, but application of systemic compounds could still result in a low, but continuous long-term exposure for pollinators with limited decline.

1. Introduction

Pollen and nectar are important food sources for pollinators and many other beneficial insects (Carreck and Williams, 1997; Thom et al., 2018; Thorp, 2000). The assessment of potential risks from oral pesticide exposure is therefore a crucial factor in pollinator risk assessments (e.g. APVMA, 2019; Cham et al., 2017; EFSA, 2013, 2023; USEPA, 2014). For higher-tier risk assessments, crop and compound-specific residue data that reflect a certain use or exposure scenario are required (EFSA, 2023; USEPA, 2014). The revised EFSA guidance document on the risk assessment of plant protection products on bees (EFSA, 2023) outlines a framework for residue field trials that aims to provide suitable conditions to reliably assess residue data in pollen and nectar (Annex B of the guidance document, *Recommendations for higher tier exposure studies*). These proposals aim to assess the spatial variation of the peak concentration of an active ingredient (AI) in nectar and pollen produced by a target crop, following a certain representative use (EFSA, 2023). For residue studies in food and feed the worst-case assumption of the Good Agricultural Practice use (Critical GAP rate) is defined as the highest application rate and highest possible number of applications, applied closest to harvest according to the product label (EU, 2005; OECD, 2009). Accordingly, the worst-case scenario for pollinator exposure has been defined as application during flowering and additional data has been reviewed in the new guideline to support this assumption (EFSA, 2023). Nevertheless, the data available pertaining to residues in pollen and nectar is still limited to a few AIs and crops, mainly neonicotinoids

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and oilseed rape (Gierer et al., 2019; Kyriakopoulou et al., 2017; Zioga et al., 2020). Therefore, the knowledge about which factors govern residue levels and dissipation rates in pollen and nectar is limited and the occurrence of residue peaks and decline from application pre-flowering are still not well understood.

Consequently, there is uncertainty whether study designs and proposed GAPs for field residue studies reflect worst-case exposure for pollinators. Furthermore, the limited data and understanding impede precise extrapolations to be made between different crops, AIs, and application types, and currently the prediction of residue levels in pollen or nectar is not feasible. Therefore, a high number of AIs, crops, and application scenarios must be considered in field trials to obtain realistic residue values for risk assessments, which requires significant expenditure and resources.

A better understanding of the formation and dissipation of pesticide residues in bee-relevant matrices could help to identify parameters that influence residue accumulation in these specific matrices, facilitate reliable data generation, and identify risk mitigation measures. Such knowledge could then be considered in the design and conduct of field trials, with the aim of providing more accurate and realistic residue values in pollen and nectar that are protective for pollinators. The identification of patterns and relationships of residues within the plant and between different plant species may permit the development of methods for estimation of residue levels for diverse AIs and cultivation methods.

This study aimed to investigate residue dynamics in floral matrices and other plant parts over an extended period and to improve understanding of the processes behind residue accumulation and decline in pollen and nectar. Courgette (also known as zucchini) (*Cucurbita pepo* L.) was used as a model plant because its flowers produce abundant amounts of pollen and nectar, which are required for the chemical analysis of pesticide residues (Gierer et al., 2019; Nepi et al., 2011; Vidal et al., 2006). *Cucurbita* sp. has been used in previous studies to investigate pesticide residues in pollen and nectar following different treatment regimens (Azpiazu et al., 2023; Dively and Kamel, 2012; Obregon et al., 2022; Stoner and Eitzer, 2012; Willis Chan et al., 2019); however, these studies focused on neonicotinoids or multi-residue analysis and data were pooled over the entire sampling periods, which did not facilitate detailed insights into residue dynamics.

In order to increase the range of knowledge of residue behaviour beyond the responses of neonicotinoids, a systemic (cyprodinil) and a contact (fludioxonil) fungicide were chosen for this study, which allowed the investigation of AIs with varying modes of action and different physicochemical properties). Both compounds are broad-spectrum fungicides, which are widely used to protect cereals, fruits, vegetables, and ornamentals from fungal pathogens as foliar spray applications or as seed and post-harvest treatments (EFSA, 2006; 2007; Lewis et al., 2016). Fungicides are considered to be less harmful to bees and can therefore be applied during flowering in many crops, including *Cucurbita* sp. (De Goes et al., 2008; McGrath and Staniszewska, 1996; Schaeffer et al., 2017; Silva-Junior et al., 2014). A recent review of the potential for exposure of bees to fungicides concluded that those AIs that most readily contaminate pollen and nectar pose the greatest potential threat (Rondeau and Raine, 2022). Adverse effects on pollinator health or synergistic effects with insecticides have been reported for a range of fungicides in recent years (Bernauer et al., 2015; Brandhorst et al., 2019; Cang et al., 2023; Carneiro et al., 2020; Cedergreen, 2014; Degrandi--Hoffman et al., 2015; Fisher et al., 2021; Iverson et al., 2019; Pineaux et al., 2023; Thompson et al., 2023; Walker et al., 2022), which has emphasised the need for risk assessments for these compounds (Cullen et al., 2019).

We investigated how residue levels vary between different plant matrices of courgette following a foliar application at an elevated application rate (approximately twice the highest permitted application rate following GAP, to ensure residue detectability) with a systemic and contact AI during flowering. These findings were used to develop an understanding of which processes determine the residue levels of the contact and systemic AI in pollen and nectar, and whether there is translocation of the systemic AI, cyprodinil, from leaves to pollen and nectar.

2. Material and methods

2.1. Growing conditions

Two experiments were conducted in glasshouses at the University of Reading, UK (51° 26′ 12″ N, 00° 56′ 33″ W). For both experiments, courgettes (*Cucurbita pepo* var. cylindrical, "All Green Bush"; Moles Seeds Ltd, Colchester, UK) were sown into germination trays containing compost (Clover Pot Compost; Clover Peat, Dungannon, Ireland) and the seedlings were re-potted after two weeks into 3-L pots containing the same compost type. An automatic drip irrigation system was set up and the plants received an all-purpose NPK fertiliser (Solufeed 1:1:1; Solufeed Ltd, Chichester, UK) two and four weeks after sowing. Insect pests were controlled with biological measures [yellow/blue sticky traps (Fargro Ltd, Arundel, UK) and predatory mites (*Amblyseius cucumeris*; Bioline AgroScience, Little Clacton, UK)]. The first experiment, which aimed to investigate residue levels in different courgette matrices following an application with two different AIs, was performed in January/February of 2020 ("Baseline Experiment"). Artificial lights with a 14-h light period were used, and the glasshouse was kept at a minimum temperature of 20 °C. The second experiment, which aimed to investigate the translocation of a systemic AI to pollen and nectar, was performed in August/September ("Translocation Experiment"). In this experiment, no additional lighting or temperature regulation was required, the average temperature was 17 °C at night and 28 °C in the day, during the sampling period.

When the plants had developed only 2–3 true leaves, the first green flower buds were already visible in both experiments (see Fig. S1 for images of all plant parts). Despite the complex morphology of courgettes, the plants had a very similar structure and grew at similar rates within each experiment. In both experiments, the flowers opened 6 weeks after they had been sown. In the translocation experiment, the female flowers opened first followed by the male flowers, which produce pollen, a week later. At least 1–2 male flowers were produced per plant on each day. In contrast, courgettes in the baseline experiment developed male and female flowers simultaneously, and on average only 0.7 male flowers per plant opened each day. Because the baseline experiment was carried out during the winter months, the lower average temperature could be responsible for the simultaneous, but reduced production of male and female flowers (Nitsch et al., 1952; Wien, 1997). In addition to the blooming flowers, plants in both experiments had 8–9 green buds in different developmental stages on their main stem when the foliar spray application was performed.

2.2. Pesticide application and experimental design – baseline experiment

When the plants exhibited 8–10 leaves and at least 80% of the plants had developed male flowers, pesticides were applied with a hand-held compression sprayer (CP5; Cooper Pegler, Villefranche, France). Ten courgette plants were gathered into treatment plots. Due to the complex structure of the courgette plants, overlap of the leaves could not be avoided but was kept to a minimum. Foliar sprays were applied to each individual plot in the morning when the male flowers were fully open. Each plot received either a treatment with 750 g AI ha^{-1} cyprodinil (Chieftain WG 50), 500 g AI ha^{-1} fludioxonil (Cannonball WG 50) (both AIs provided by Syngenta Crop Protection AG, Munchwilen, Switzerland) or was left untreated as a control plot. The chosen application rates corresponded approximately to twice the cGAP rate (highest permitted application rate following GAP), to ensure residues could be detected at levels above the limit of quantification (LOQ; 0.01 mg kg^{-1}) in pollen and nectar. The applications were made in 150 L water ha^{-1}

and calculations were based on a plant density of 11,000 plants ha^{-1}. Overall, four replicate plots were prepared for each treatment. As soon as the spray solution had dried (approximately 1 h after application), the plots with different treatments were arranged in a fully randomised block design in the glasshouse (Fig. S2).

2.3. Pesticide application and experimental design – translocation experiment

In the translocation experiment, the AI cyprodinil was applied only to the leaf surfaces of each individual courgette plant rather than overhead to the entire plot. Therefore, a small, handheld spray bottle was used for the application, which allowed a metered dose of 0.12 mL per spray pump. A spray solution at a concentration of 68 mg AI mL^{-1} was prepared and each individual courgette plant received 8 pump sprays of the solution onto the leaves. This was the equivalent amount of AI that an individual plant had theoretically received in the baseline experiment. Older and younger leaves were sprayed and the flowers and green buds in the middle of the plant were protected with a plastic sheet to avoid contamination. The plants were sprayed in the late afternoon. After the spray solution had dried, the plants were distributed across the glasshouse in 5 replicate plots consisting of 10 plants each, to compensate for any small environmental differences within the glasshouse.

2.4. Sample collection

In the baseline experiment, the first samples of pollen, nectar, anthers, flowers, and leaves were taken on the day of the pesticide application (0 days after application, 0 DAA) as soon as the spray solution had dried, which was approximately 1 h after application. Further samples were taken each morning for the subsequent 13 days. In the translocation experiment, only pollen, nectar, and anthers were collected. The first samples were also taken on 0 DAA; however, they were taken before the AI had been applied to serve as control samples. Translocation from the leaves to flowers was not expected to occur shortly after application, therefore, the first treated samples were taken on 1 DAA. The sample collection continued over the following 10 days in the late morning. In both experiments, on each sampling day, all male flowers growing in a replicate plot were cut and immediately processed (Fig. S3). The petals were removed, and the pollen was gently scraped into 2-mL Eppendorf tubes using a plastic strip (pot label). Nectar was transferred from the base of each flower into 1.5-mL Eppendorf tubes using a 100-μL micropipette. Flowers and anthers from the baseline experiment were stored separately in resealable plastic bags. This was done until all flowers from all treatments were collected and processed. All tools and gloves were exchanged between each replicate to avoid contamination and samples were stored at −20 °C. In addition to pollen, nectar, flowers, and anthers, leaf samples were taken daily in the baseline experiment. Leaves in different developmental stages were collected across each replicate plot and pooled to obtain a representative sample.

2.5. Chemical analysis

All samples were analysed using the method described in supplementary material 1, which met the requirements outlined in guidance document SANTE/12682/2019 (European Commission, 2019). In short, homogenised subsamples of 0.03 g (nectar), 0.05 g (pollen), 0.15 g (anthers), 0.50 g (leaves), and 1.00 g (flowers) were weighed into appropriately sized tubes along with lysing beads. The AIs were extracted twice using acetonitrile (ACN) as the extraction solvent, a tissue homogeniser (FastPrep 5G; MP Biomedicals Cambridge, UK), ultrasonication, and centrifugation. The extracts were diluted with ultrapure water into vials and analysed against matrix-matched standards by LC-MS/MS (6410 series, Agilent Technologies, Santa Clara, California, US). Each sample batch contained at least one control sample from the experiment, one solvent control, and three to five fortified samples at two concentrations as a quality control. Control samples of all plant matrices were free from contamination and the fortified control samples afforded acceptable recoveries (70–110%).

2.6. Statistical analysis

The statistical analysis was performed using R (version 4.0) (R Core Team, 2020) and focused on the recorded residues in different plant matrices. The purpose of control samples was to confirm that cross-contamination did not occur; therefore, these data were not included in the analysis. For the evaluation of residue levels and dissipation trends in courgette, a linear mixed model was fitted for each AI used in the baseline experiment, with matrix and DAA as fixed effects and replicate as a random effect. The diagnostic plots showed a skewed distribution of the residues. Therefore, a Box–Cox transformation was applied to improve the assumptions of normality and equal variances (Box and Cox, 1964; Gurka et al., 2006). An analysis of variance (ANOVA) with Satterthwaite's approximation and pairwise comparisons of estimated marginal means with Tukey correction ($\alpha = 0.05$) was performed to detect significant differences between plant matrices and DAA. The model summaries were used to evaluate trends in each matrix. A similar approach was used to evaluate the translocation of cyprodinil to pollen and nectar in the translocation experiment. For the comparison of residue levels between cyprodinil and fludioxonil, the residue data was transformed to Residue Unit Dose (RUD), which is the concentration of AI in the matrix (mg kg^{-1}) at an application rate of 1 kg AI ha^{-1} (EFSA, 2023). Again, a linear model with Box–Cox transformation was fitted and analysed using a similar approach as described for the individual AI.

3. Results

3.1. Residue levels and dissipation trends of cyprodinil in various courgette matrices following a foliar spray application

Cyprodinil residues were detectable in treated leaves, flowers, anthers, pollen, and nectar of courgette plants above the limit of quantification (0.01 mg kg^{-1}) over a period of 14 days (Fig. 1). All control samples were free from contamination. The level of residues detected depended on the plant matrix ($F(4, 200.1) = 5754.1$, $P < 0.001$), the day after application (DAA) ($F(13, 200.1) = 158.8$, $P < 0.001$), and their interaction ($F(52, 200.0) = 25.0$, $P < 0.001$). Relative standard deviations (RSD) calculated for each DAA and matrix ranged from 14.3 to 75.2% in anthers, 20.9–54.0% in flowers, 6.5–33.6% in leaves, 16.9–86.1% in nectar and 16.11–81.7% in pollen. Detailed residue value data can be found in Table S1 or in Gierer (2022).

On the day of the application the highest cyprodinil residues were found in the leaf samples of courgette plants ($P < 0.001$) (Fig. 1). The residues in leaves declined only marginally from 0 to 13 DAA ($P = 0.049$) and hence, were higher than in any other matrix over the course of the entire sampling period ($P < 0.002$). Cyprodinil residues detected in courgette flowers were significantly lower than in leaves on 0 DAA and decreased continuously throughout the sampling period by 99.98% ($P < 0.001$). The highest dissipation rate in flowers was observed from 0 to 1 DAA, within that day cyprodinil residues declined by 92.74% ($P < 0.001$). Residues in pollen and nectar were significantly lower than those in flowers on 0 DAA ($P < 0.001$) and residues detected in both matrices did not differ statistically on the day of the application ($P = 0.992$). Similarly to the flowers data, a pronounced decline in residues was observed from 0 to 1 DAA in pollen and nectar. However, cyprodinil residues in pollen did not decline any further during the sampling period and remained at a consistent level on all subsequent DAA ($P \geq 0.456$). Cyprodinil residues in nectar were significantly lower than in any other matrix ($P < 0.001$) on all days after 0 DAA. The LOQ of 0.01 mg kg^{-1} was only exceeded on 0, 1, 3 and 5 DAA. There were no significant

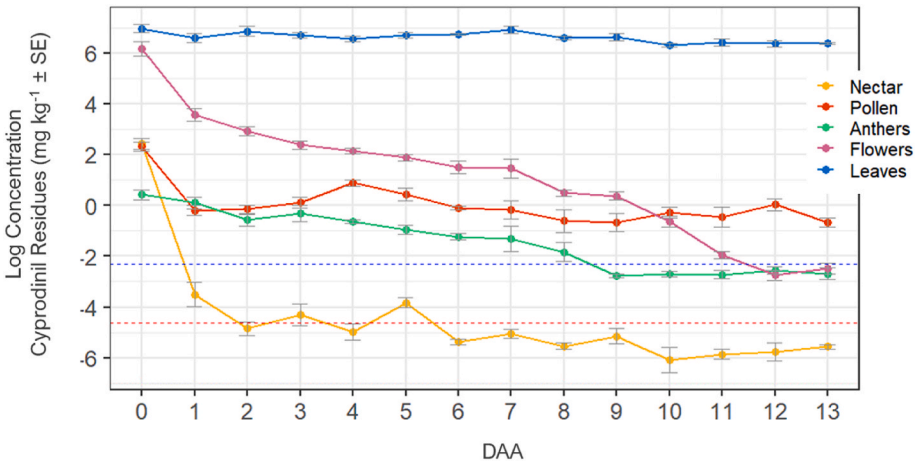


Fig. 1. Mean log-transformed cyprodinil residues (mg kg^{-1}) in courgette leaves, flowers, anthers, pollen, and nectar. Samples were collected on 0–13 days after application (DAA). The grey bars show the ±standard error (SE) of the mean. The red dashed line represents the (log-transformed) LOQ of pollen, anthers, and nectar (0.01 mg kg^{-1}), the blue dashed line represents the 0.1 mg kg^{-1} level.

differences for any pairwise comparisons of DAA in nectar between 2 and 13 DAA ($P \geq 0.137$). The lowest cyprodinil residues on 0 DAA were found in anthers ($P < 0.001$); residue levels declined significantly over the course of the sampling period ($P < 0.001$). The decline of cyprodinil residues in anthers was more pronounced than in pollen ($P = 0.017$), but slower than in flowers ($P = 0.002$). Due to the different dissipation trends, residues in pollen were on a similar level to anthers on 1–3 DAA ($P \geq 0.682$), but significantly higher on the following sampling days ($P < 0.001$). Furthermore, the cyprodinil levels were lower in pollen than in flowers from 0 to 9 DAA ($P < 0.009$), but significantly higher again than in flowers towards the end of the sampling period ($P < 0.001$).

3.2. Residue levels and dissipation trends of fludioxonil in various courgette matrices following a foliar spray application

Fludioxonil was detected in all treated plant matrices on all DAA at levels above the LOQ, except for residues in nectar and some DAA in pollen (Fig. 2). All control samples were free from contamination. Fludioxonil residues varied depending on the plant matrix (F (4, 191.2) = 5569.5, $P < 0.001$), the DAA (F (13, 191.1) = 148.3, $P < 0.001$), and their interaction (F (48, 191.1) = 16.9, $P < 0.001$). The RSDs ranged from 19.2 to 121.0% in anthers, 16.7–50.6% in flowers, 16.0–46.3% in leaves, 20.6–105.2% in pollen and 16.7–120.1% in nectar. Detailed residue value data can be found in Table S2 or in Gierer (2022). The highest fludioxonil residues were detected in courgette leaves ($P < 0.001$), which remained consistent between most DAA of the sampling period with no significant daily variations ($P \geq 0.093$). Fludioxonil residues in flowers were higher than in pollen or anthers on all DAA ($P < 0.001$). The most pronounced decrease of 93.7% was detected from 0 DAA to 1 DAA ($P < 0.001$), and the residue levels in flowers decreased continuously through the sampling period ($P < 0.001$). The residue levels found in anthers were substantially lower than in pollen on 0 DAA ($P < 0.001$); however, the decrease from 0 to 1 DAA was less pronounced than in pollen ($P < 0.001$). Fludioxonil continued to decrease slowly in anthers on the following DAA, but the residues remained higher than in pollen on the majority of days ($P < 0.002$) until a significant decrease of 92% was observed in anther residue levels between 9 and 11 DAA ($P < 0.001$). Fludioxonil residues in pollen decreased significantly by 98.8% from 0 to 1 DAA and again by 82.5% from 4 to 5 DAA ($P < 0.001$ and $P = 0.032$, respectively), but there were no further significant differences between the other DAA ($P \geq 0.596$) and they remained close to the LOQ for the remaining sampling period. Fludioxonil residues in nectar were not significantly different from those found in pollen on 0 DAA ($P = 0.913$) but decreased significantly below the LOQ of 0.01 mg kg^{-1} on 1 DAA ($P < 0.001$) and could not be detected at all from 10 DAA onwards.

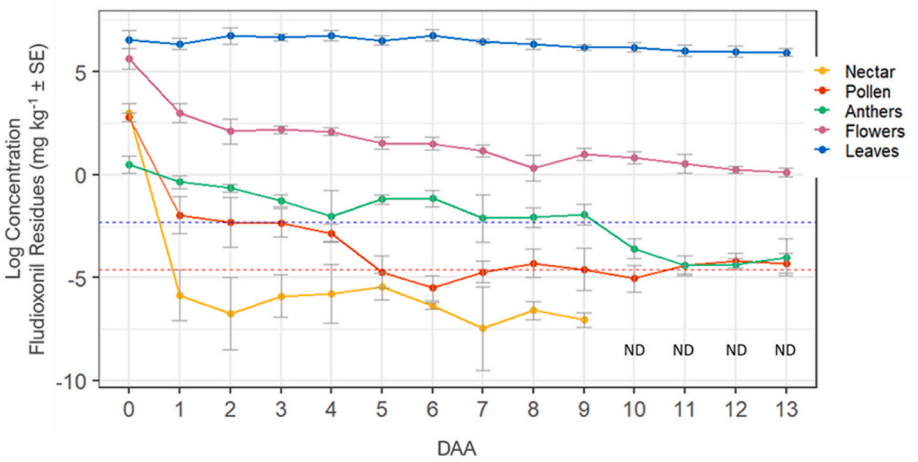


Fig. 2. Mean log-transformed fludioxonil residues (mg kg^{-1}) in courgette leaves, flowers, anthers, pollen, and nectar. Samples were collected on 0–13 days after application (DAA). The grey bars show the ±standard error (SE) of the mean. The red dashed line represents the (log-transformed) LOQ of pollen, anthers, and nectar (0.01 mg kg^{-1}), the blue dashed line represents the 0.1 mg kg^{-1} level. There were no fludioxonil residues detected in nectar on 10–13 DAA (ND).

3.3. Comparison of cyprodinil and fludioxonil residues (RUD values) between different matrices

There were significant differences between the RUD values (Fig. 3) for the two AIs (F (1, 394.0) = 70.6, $P < 0.001$), as well as between matrices (F (4, 394.1) = 11514.7, $P < 0.001$), DAA (F (13, 394.1) = 294.7, $P < 0.001$) and all the two- and three-way interactions (see Table S3 for full ANOVA output). The RUD values in courgette leaves were similar after the application of fludioxonil and cyprodinil on most DAA ($P \geq 0.079$) (Fig. 3). However, due to slightly different daily trends at the beginning of the sampling period, fludioxonil RUD values were approximately 1.5 times higher than the cyprodinil RUDs on 3, 4 and 6 DAA ($P \leq 0.031$). RUD values in courgette flowers were also similar between AIs in the first half of the sampling period, with no significant differences between the fludioxonil and cyprodinil residues from 0 to 8 DAA ($P \geq 0.139$). However, at the end of the sampling period (9–13 DAA), cyprodinil RUDs declined more quickly than fludioxonil RUDs and were accordingly significantly lower ($P < 0.001$). RUD values in anthers were also similar between AIs on the majority of DAA. Significant differences could only be observed on 3, 9, 11, and 12 DAA ($P \leq 0.030$). Fludioxonil residues in anthers declined more quickly at the end of the sampling period and were therefore significantly lower than cyprodinil on 10–13 DAA ($P \leq 0.016$).

In contrast, the RUD values of cyprodinil and fludioxonil in pollen samples were significantly different on all investigated DAA. Fludioxonil RUDs were twice as high as cyprodinil RUDs on the day of the application (0 DAA) ($P = 0.001$), but on 1–13 DAA the cyprodinil RUDs were on average 45 times higher than the fludioxonil RUDs ($P < 0.001$). In nectar, the fludioxonil RUDs were 2.5 times higher than the cyprodinil RUDs on 0 DAA ($P < 0.001$), but cyprodinil RUDs were higher on 1, 2, 7, and 9 DAA ($P \leq 0.031$). However, in nectar, even the fludioxonil RUDs, which account for the lower application rate of the fludioxonil application, were below the LOQ on the majority of DAA.

3.4. Translocation of cyprodinil to pollen and nectar

Cyprodinil residues were found in pollen and nectar samples on all DAA following a targeted application to courgette leaves only. Samples collected on 0 DAA, just prior to the application, were free from residues. Significant differences in residue levels were detected between residues in pollen and nectar (F (1, 76) = 158.9, $P < 0.001$). Furthermore, the two-way interaction between matrix and DAA was significant (F (9, 76) = 2.1, $P = 0.036$), but the main effect, DAA, did not have a significant influence on the residue levels (F (9, 76) = 2.0, $P = 0.051$). Cyprodinil residues in nectar were significantly lower than in pollen on all DAA ($P < 0.01$) (Fig. 4). The highest mean values in nectar were detected on 5, 7, and 8 DAA, but on the majority of sampling days the values were below the LOQ. In pollen, the highest mean values were detected on 2, 3, 5, and 8 DAA, but these data showed considerable variability and no significant differences were observed between these DAA and the pollen samples on the other DAA ($P \geq 0.349$). Residues found in both matrices exhibited large relative standard deviations (Table S4), which exceeded 100% on several days, indicating a large variability.

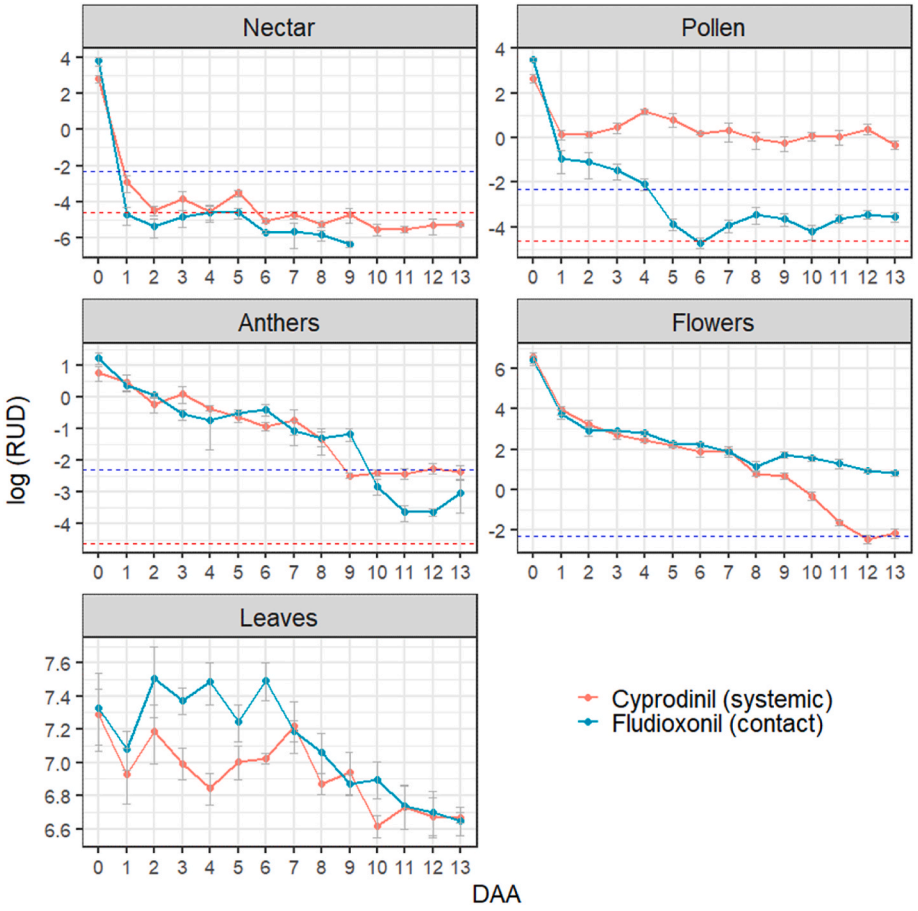


Fig. 3. Comparison of mean cyprodinil and fludioxonil residue unit dose (RUD) values found in different courgette matrices at 0–13 days after application (DAA). The RUD values were log-transformed to account for the wide range of RUD values within one matrix. Grey bars represent the standard error of the mean on each DAA. The red dashed line represents the (log-transformed) LOQ of pollen, anthers, and nectar (0.01 mg kg^{-1}), the blue dashed line represents the 0.1 mg kg^{-1} level.

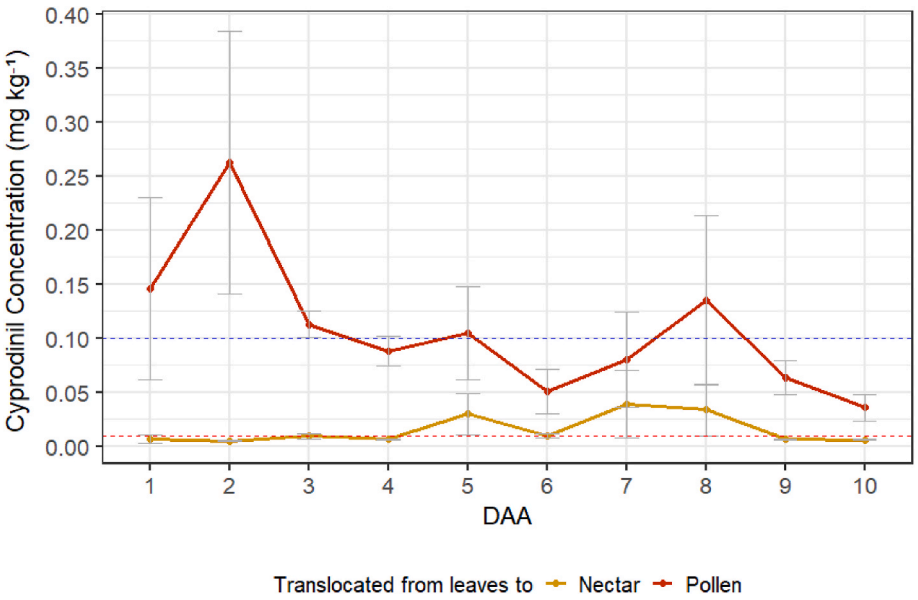


Fig. 4. Comparison of mean cyprodinil residues (mg kg^{-1}) translocated from courgette leaves into pollen (red) and nectar (yellow) on 1–10 days after application (DAA). Samples on 0 DAA were collected before the application and were free from any contamination. Grey bars represent the standard error of the mean. The red dashed line represents the limit of quantification (LOQ = 0.01 mg kg^{-1}), the blue dashed line represents the level of 0.1 mg kg^{-1}.

4. Discussion

4.1. Residue levels of cyprodinil and fludioxonil in various courgette matrices following a foliar application at an elevated application rate during flowering

The experimental design chosen for the present study was demonstrated to be effective for investigating the residue dynamics in different plant matrices over an extended period. Due to the elevated application rates, which were not representative of typical use patterns, residues of both AIs in courgette leaves were higher and did not decline significantly compared to residues studied in other crops (Cabizza et al., 2007; EFSA, 2006; 2011; Garau et al., 2002). However, there have been no comparable studies published on cyprodinil and fludioxonil residues in courgettes or other cucurbits. Nevertheless, residues of both AIs in nectar declined to the LOQ of 0.01 mg kg^{-1} quickly after the application, demonstrating that elevated application rates were required for the investigation of residue dynamics in floral matrices.

Generally, residue levels decreased in the following order: leaves > flowers > anthers > pollen > nectar, throughout the sampling period, except for cyprodinil residues in pollen. This is in line with a study in squash by Dively and Kamel (2012), who detected higher residues in leaves than in pollen and nectar following foliar and soil applications of neonicotinoids, although the differences between matrices varied with AI and treatment type. Equally, Stoner and Eitzer (2012) found the highest neonicotinoid residues in squash when the entire plant was analysed, but the comparison of residues in flower bases and anthers yielded varying results, depending on the treatment, the AI, and the year. However, in both studies, the residues were pooled over the entire sampling period.

The temporal separation achieved by using daily collections and a model system in the present study demonstrated that the residue decline rates and residue dynamics changed over time in each plant matrix, and differed between the systemic and contact AI. For example, on the day of the application, plant matrices that were equally exposed to the spray application due to their position on the plant, contained comparable residue levels (in RUD), such as pollen and nectar or flowers and leaves. In contrast, floral matrices collected later in the sampling period were not directly exposed to the AI, and thus were subject to translocation and accumulation processes, resulting in varying residue levels across floral matrices and AIs. This suggests that the different physicochemical properties of each AI affected the residue development differently in each matrix over time which is discussed below for each matrix investigated.

In general, it is understood that contact AIs tend to remain around the application area but penetrate the plant tissue to a certain extent, while systemic compounds penetrate the plant tissue at the application site, but are also translocated through the vascular system of the plant (Hsu et al., 1990; Sicbaldi et al., 1997; Wang and Liu, 2007; Zhang et al., 2017; Zhang et al., 2018). Residues in floral matrices collected in the baseline experiment following a spray application during flowering could therefore originate from a) direct exposure as shown on 0 DAA, b) lateral movement directed by sorption and partitioning of AIs across lipophilic plant parts following an equilibrium gradient and by distribution of the aqueous phase in the plant (Briggs et al., 1982; Briggs et al., 1983; Collins et al., 2006), and c) remote translocation from other plant matrices such as leaves or stems, if the AI's properties allow systemic translocation. Residues in pollen or nectar collected in the translocation experiment could only originate from remote translocation. This framework could be used to improve the understanding of residue dynamics and related processes in each floral matrix over time.

4.2. Residue dynamics in flowers and anthers

Despite the different physicochemical properties of both compounds, RUD levels and dissipation trends of cyprodinil and fludioxonil were very similar in flowers and anthers in the baseline experiment until 9 DAA. During the application, 8–9 green flower buds on each plant were directly exposed to the spray solution. This indicates that both compounds were equally translocated laterally from the external layers of the flower bud and adjacent tissues into the flowers and anthers.

Flower buds emerging on the plant after the day of the application (flowering after 8–9 DAA) were not directly exposed to the AIs and showed a different trend in residue development than earlier buds. Fludioxonil residues in flowers declined steadily, while fludioxonil residues in anthers decreased significantly after 9 DAA. The contact AI fludioxonil is known to be persistent in plant tissue with the potential to bioaccumulate due to its high lipophilicity (EFSA, 2007; Gong et al., 2020; Lewis et al., 2016; Pereira et al., 2016). Such compounds are also partitioned along plant membranes, which could explain the declining

trend in the deeper anther tissue after 9 DAA.

Cyprodinil residues showed a reverse trend in flowers and anthers compared to fludioxonil. It could be expected that cyprodinil, as a xylem-translocated compound, would be significantly translocated into flowers and anthers from lower plant parts because the expansion of flowers depends on a high influx of water (De la Barrera and Nobel, 2004). Furthermore, the translocation experiment showed that translocation into pollen and nectar did occur. However, cyprodinil residue levels in flowers declined continuously after 9 DAA, while residues in anthers remained relatively consistent. Cyprodinil has a lower lipophilicity and higher solubility than fludioxonil and is therefore more easily translocated and diluted within the entire plant (Garau et al., 2002; Lewis et al., 2016), which could explain a less pronounced lateral movement of the AI from the plant surface into the flower tissue compared to fludioxonil beyond 9 DAA. Furthermore, De la Barrera and Nobel (2004) hypothesised that the turgor in flowers is provided by the phloem and not the xylem, because the water deficit in flowers would not be large enough to maintain a constant water supply from the xylem. This would impede the translocation of a xylem mobile AI into flowers.

Cyprodinil residues in anthers were consistently lower than in flowers. However, unlike in flowers, cyprodinil residues in anthers did not decline significantly beyond 9 DAA and levels in both matrices were similar by the end of the sampling period. Anthers have been shown to have the highest sink strength in flowers (the ability of an organ to import assimilates for its growth, development, and maintenance (Chamont, 1993) and a high carbohydrate content during pollen formation (Clément and Audran, 1995, 1999). Accordingly, the nutritional supply of anthers must be ensured by a constant stream of nutrients and water, which could provide a route for the translocation of systemic compounds from distal plant parts (e.g., leaves) into anthers. Stoner and Eitzer (2012) found higher residues of two systemic (soil-applied) pesticides in squash anthers compared to the flower bases. This effect was only observed in one year, and samples were taken before anthesis and pooled from the entire sampling period in their study. Nevertheless, these findings emphasise anthers as a potential sink for systemically translocated residues and highlight their potentially specific role in residue translocation processes.

4.3. Residue dynamics in courgette pollen

Residues of fludioxonil and cyprodinil in pollen followed different dynamics than were observed in flowers or anthers. Pollen is produced within the anther and hence is more protected by residues diffusing from the plant surface through anthers. The less mobile AI fludioxonil declined very quickly in pollen and residue levels were below those in anthers, which is consistent with the assumption that the highly lipophilic AI fludioxonil is partitioned and bound along cell membranes (Price, 1990; Sicbaldi et al., 1997; Stevens et al., 1988). On the contrary, the systemic compound cyprodinil did not decline significantly during the sampling period from 1 DAA onwards and cyprodinil RUDs in pollen were substantially higher than fludioxonil RUDs, suggesting that in addition to the lateral movement, cyprodinil was translocated from distal plant parts. Additionally, cyprodinil residues in pollen were higher than in anthers from 5 DAA onwards, implying that cyprodinil had been directly translocated into pollen, bypassing anther tissue.

Anthers consist of several cell layers and pollen develops in a cavity known as loculus. Inside the loculus, the tapetum secretes the locular fluid, which contains nutrients for pollen development and growth, such as polysaccharides, pectins, proteins, and lipids (Bedinger, 1992; Carrizo García et al., 2015; Clément and Audran, 1995, 1999; Pacini, 2000; Pacini and Dolferus, 2016). Nutrients are supplied by the vascular bundle, which leads through the filament into anthers, but are also provided by external cell layers of the anther (Clément and Audran, 1999; Pacini and Dolferus, 2016). It was shown that pollen is closely related to its surrounding environment at all stages of development, forming an equilibrium with the developing pollen cells (Carrizo García et al., 2015). Thus, a mobile compound could be transferred into the sporophytic anther tissue from inside the plant through the anther's vascular bundle. Avila-Sakar et al. (2003) showed that the early stages of pollen development in *Cucurbita* species begin when the buds are barely visible in the leaf axis. The translocation and diffusion of residues within the plant could therefore affect final residue levels at anthesis from a very early stage of flower bud development. Hence, residues in pollen could originate from a long-term accumulation, resulting in noticeable residue levels, even if the application is performed weeks before anthesis, as shown in the baseline experiment and other studies that used seed dressings and soil applications at planting (Dively and Kamel, 2012; Stoner and Eitzer, 2012).

The translocation experiment demonstrated that cyprodinil can be translocated from distal plant parts into pollen, although an increased accumulation over time was not observed. However, residue partitioning from the plant surface of flower buds could not occur in this experiment, because only individual leaves were sprayed. Translocation effects were therefore not masked by any other movements of AI. Consequently, the residues in pollen were substantially lower in the translocation experiment than in the baseline experiment. Furthermore, cyprodinil is translocated in the xylem of plants and thus, would follow only an acropetal movement to plant parts with a water deficit (Avila-Sakar et al., 2003). Flower shoots on the courgette plants used for the translocation experiment were located below younger leaf shoots, but above older shoots on the main stem of the plant. Therefore, cyprodinil was presumably not translocated from every leaf of the plants, which may have resulted in lower residue levels in pollen.

4.4. Residue dynamics in courgette nectar

Nectaries in courgettes are located at the base of the flower filament (Fig. S3) (Nepi et al., 1996b; Pacini et al., 2003) and are therefore connected to similar plant tissues as the anther filament, suggesting that residue levels in nectar could be expected to be similar to those in anthers or pollen. Residues of fludioxonil were detected in nectar at the beginning of the sampling period but decreased below the LOQ within one day. After 9 DAA fludioxonil could not be detected at all in nectar. These results correspond with observations of fludioxonil residues made in the pollen samples, although the nectaries may be even better protected from diffusion of the AI through the membranes from surrounding plant tissue. Similar observations were made by Raimets et al. (2020) and Rondeau and Raine (2022) who mainly found residues of systemic and hydrophilic AIs in honey, but limited evidence for residues of highly lipophilic, contact AIs. In contrast to fludioxonil, cyprodinil was detected in nectar throughout the sampling period. However, cyprodinil residues in nectar were substantially lower than in pollen, which is in line with other residue studies in *Cucurbita* sp. using systemic AIs (Dively and Kamel, 2012; Stoner and Eitzer, 2012). Potential translocation of cyprodinil into the nectaries seems therefore to be less pronounced than to pollen.

The difference between fludioxonil and cyprodinil residues in nectar was considerably smaller than was observed between both AIs in pollen, suggesting that residue translocation into nectar, or AI secretion in nectaries, may follow a different process. This assumption is supported by the translocation experiment, which yielded substantially lower cyprodinil residues in nectar than in pollen following transport from distal leaves. Although in this experiment peak residue levels were observed in pollen and nectar on similar days, the significant two-way interaction between matrix and DAA suggests that the translocation rates to pollen and nectar differed.

The nectar-producing parenchyma in *Cucurbita* is only supplied by phloem, which branches near the nectar-secreting stomata, while xylem vessels end at the base of the parenchyma (Dmitruk and Weryszko-Chmielewska, 2013; Nepi et al., 1996b). Therefore, the possibility exists that xylem-translocated, and nonpolar AIs, such as cyprodinil, may not reach the nectar-secreting tissues directly, but are

partitioned along cell membranes of the parenchyma inside the plant.

Another possibility is that residues in nectar are generally more diluted than in pollen or anthers. Nectar is secreted in courgettes through modified stomata that have lost the ability to close (Pacini et al., 2003). Starch is stored in the nectar-producing parenchyma several days before anthesis and is hydrolysed before the start of secretion into soluble sugars, which causes a rapid influx of water into the nectary and initiates nectar secretion (Dmitruk and Weryszko-Chmielewska, 2013; Nepi et al., 1996a; Nepi et al. 2011; Nepi et al. 1996b; Pacini et al., 2003). Nectar is secreted over a period of several hours in courgette and up to 170 μL can be collected from a single flower per day, but nectar secretion and nectar sugar content vary during anthesis (Nepi et al., 2001; Vidal et al., 2006). In the baseline experiment, nectar was always collected at a similar time during the day, but nectar volumes or sugar content as indicator of dilution were not measured. Therefore, it is unclear whether dilution influenced nectar residue levels and whether there was a relationship between residue expression in nectar and pollen.

Overall, higher variability in residue values of pollen and nectar was observed compared to variations of residues in flowers or leaves. Residues that originated solely from translocation, exhibited high variation, particularly in the translocation experiment. This can partially be explained by residue levels below the LOQ, i.e. they could not be measured accurately (Ambrus, 2006). However, the high variability observed in these specific matrices indicates that complex processes were involved in residue formation, which varied depending on the matrix and AI investigated.

5. Conclusions

Residues of the contact AI, fludioxonil, declined quickly in pollen and nectar but were detected in flowers or anthers over a longer period. This suggests that if the nectaries of a plant like courgette are well protected within a flower bud, there is a low likelihood that an immobile compound will enter nectar if applied before bud formation. Consequently, the risk of oral exposure for pollinators is considerably reduced from the use of contact AIs on crops with comparable morphology, even if they are applied on flower buds. In contrast, the results obtained under fully controlled conditions in this study, suggest that the application of systemic compounds, such as cyprodinil, could result in a potentially low, but continuous long-term exposure with limited residue decline in pollen for pollinators in a field scenario. Consequently, pollinators are exposed to varying residue levels during the anthesis period, and overall exposure may depend on whether mainly nectar or pollen is collected. These dynamics should also be considered in field trials for data generation to capture both short-term residue peaks, as well as long-term exposure for pollinators using sufficient and representative sampling points during anthesis. Nevertheless, residues in pollen remained always higher than in nectar, which could be used as a conservative, worst-case exposure estimate following foliar spray applications.

Residue formation in pollen and nectar seems to follow different processes, which was hypothesised to involve lateral movement of the compounds through adjacent plant tissue and translocation of the systemic compound from lower plant parts. The higher residues and different dynamics found in pollen suggested that a) structural differences in the vascular system led to different translocation rates into the respective plant organs and b) the chemical properties of pollen and nectar and the AI could affect residue levels. Therefore, in-depth knowledge about these relationships in a model plant such as courgette could further improve the understanding of residue formation in other plants that produce less accessible pollen and nectar.

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Declaration of competing interest

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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