Determination of the sampling size for the reliable identification of organic crops by inducing sublethal effects in beneficial insects

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Abstract. Among the main innovations in olive growing, the conversion of cultivation practices towards organic agriculture stands out. Since the organic crops are subject to the granting of economic subsidies by the Spanish Ministry of Agriculture, this has given rise to fraudulent situations, masking the use of conventional pesticides in "fake organic" crops. To investigate alternative methods, complementary to analytical chemists that can provide reliable information to discerning organic crops from those subjected to synthetic pesticides is the aim of this research. A new technique is being developed, based on the induction of sublethal effects on the beneficial insects, through the experimental application of a commercial insecticide on a small scale, in the target crop. The effect triggered by the insecticide can be monitored by means of a yellow sticky trap device, installed for each olive tree, which allows to observe deviations in its capture rate from what occurs in the absence of insecticide (control) and finally verifying if the populations of beneficial insects affected in the olive grove, whether or not they are accustomed to the toxic action of the insecticide. Obviously, in order to maintain environmental integrity in the supposed ecological target olive groves, this induction test should be applied on the smallest scale as possible, thus reducing the application area of the pesticide, which implies adjusting the sample size to a minimum, maintaining an acceptable reliability in the estimates. During the autumn of 2017, six plots of 7x7 configuration were selected in an olive grove in the province of Jaén (southern Spain), three of these were treated with Dimethoate, while the remaining were considered as control. After the treatment application, sticky yellow traps were installed (one in each of the olive trees), thus allowing a maximum of 49 repetitions per plot. Among the identified beneficial insects, the most abundant species was *Aeolothrips intermedius*. Taking this species as a reference, the results indicate that the differences between treated and control parcel were statistically significant from a minimum sample size of 17 replications onwards. In coincidence with the above, the results have allowed determining that the minimum size of the experimental target plot, to obtain acceptable population estimates (relative error of up to 10%), would require a minimum area of at least 100 m², representing a minimum number of 17 olive trees. These results provide a reasonable statistical basis for suitably adapting the methodology that allows acceptable estimates, therefore, the application of this methodology of detection of organic crops would require a very small area, equivalent to a practically insignificant number of olive trees, so it would not compromise the ecological quality of the target plantation.

1. Introduction

As in any other agricultural crop, the determination of the application of pesticides in the olive grove is based exclusively on the results of chemical analyses on plant organs. The most common procedures for
these analyses are gas chromatography [1], liquid chromatography [2; 3], liquid-gas chromatography [4] and the chemiluminescence method [5]. These methods are highly complex since the fruit matrix is mainly composed of triglycerides (98-99%) and most pesticides are fat-soluble polar compounds, making it difficult to detect [6]. Furthermore, the perishable nature of pesticide residues added to the incidence of changing climatic factors (temperature, photoperiod, radiation intensity ...) cause significant restrictions on the reliability of the results. Since organic crops are subject to the granting of economic subsidies by the Spanish Ministry of Agriculture, this has given rise to fraudulent situations, masking the use of conventional pesticides in "fake organic" crops, whose objective is to fraudulently obtain these subsidies. This has suggested the need to investigate complementary / alternative methods that provide reliable information that allows discerning organic crops from those other, subject to conventional pesticides.

Recently, a new technique is being developed, based on the induction of sublethal effects on the beneficial entomofauna, through the experimental application of an organophosphate insecticide (Dimethoate) [7; 8], and in the monitoring of the induced effect on the capture rate, using a sampling device of sticky yellow traps. The results have concluded that in crops subjected to a regular application of pesticides, the beneficial insects would present an escape reaction from the treated area. This would allow them to minimize contacts with insecticide impregnated surfaces [7-9], seeking refuge on surfaces free of the pesticide, which therefore manifests itself in an increase in its catch rate in sticky traps compared to what would be expected. In contrast, in crops subject to organic management, beneficial insect here have not been exposed to insecticides, making them much more susceptible, causing a higher mortality rate. This increased knockdown effect in organic crops manifests itself in a reduction in the capture rates of beneficial insects in the sticky traps of the insecticide-treated areas. [7]. These large differences in the behaviour of beneficial insects, depending on the application regimen of insecticides, have suggested their use as a tool for the development of a technique based on an experimental application of the insecticide on a small scale, in a small area of a crop target, with the aim of provoking a reaction in beneficial insects, and their subsequent monitoring. Its different behaviour, in olive groves of each of these types (organic or IPM) would allow providing the data to determine with precision if a target crop is being subjected to a regular insecticide application, or if it is a real organic crop.

With the aim of interfering as little as possible in the management regimen of the target olive grove (and more especially in the case of a true organic crop), the aim of this research is to improve this diagnostic methodology, especially with regard to the determination of the minimum sample size to obtain statistically reliable estimates that allow the precise identification of organic crops.

2. Materials and Methods

2.1. Description of the study area
The study was carried out in the province of Jaén (Andalusia, southern Spain) during the autumn of 2017. The olive grove used for this study is representative of the integrated pest management regime (IPM). It is located at the coordinates 37º 37'44.79 " N 3º26'32.75 " W. The olive trees of this crop are of the picual variety, of 20–30 years old, and planted at a density of 100 olive trees/hectare. In this olive grove, the growth of a vegetal cover, of approximately 2.5 m, located in the central area of the corridor between two contiguous rows of olive trees, is encouraged, which is periodically controlled by the use of a brush cutter. The objective is to reduce water competition with olive trees from the month of April when water is scarce. Among the species that form this cover, the following stand out: Lolium rigidum Gaudin, Senecio vulgaris Linneo, Poa annua Linneo, Silene colorata Poiret, Diplotaxis virgata Candolle, Muscari neglectum Gussone, Sinapis alba Linneo, Equisetum arvense Linneo, Bromus madritensis Linneo, Convolvulus althaeoides Linneo, Phalaris minor Retzius, Daucus carota Linneo, Cirsium arvense Scopoli, and Anacyclus clavatus Persoon.
Pest control is regularly carried out through the application of commercial organophosphorus insecticides, usually, Dimethoate 40%© (400g/l) (BASF) applied at a concentration of 0.1% (100cc/hl), during spring (May-June) and autumn (September-October).

2.2. Experimental set up
The study was carried out from September 27 to October 11, the period of chemical control against Bactrocera oleae in southern Spain, since according to the indications of the Regional Government the levels of the capture of adults in the sampling devices exceeded the threshold 5 adults/day.

Within the olive grove, six plots were selected, each containing 49 olive trees (7x7 configuration). By random selection, three of them were selected and treated with 40% Dimethoate at a concentration of 0.1% (v/v) by a hydraulic backpack sprayer (MATABI Evolution 16©) of 16 l capacity. A homogeneous volume of solution between 2 l and 3 l per tree, were applied on atmospheric calm conditions, ranging the wind speed from 3 to 5 km/h. The remaining three plots were considered control plots and only distilled water was applied within them.

Sticky yellow traps were used to control the response of beneficial insects after pesticide application. This type of sampling based on the movement of the insects towards the chromatic traps [7; 8; 10; 11] has shown excellent results and is appropriate for replication [7; 11; 12]. After insecticide application, one yellow sticky trap, with dimensions of 20 cm x 40 cm was placed in each tree of the 6 plots, at approximately 1.5 m from the ground, in the southern sector, in order to adapt the samplings to the microclimatic preferences of the natural enemies.

Traps were removed and renewed in two intervals of 7 days after experimental application (September 27-October 4 and October 4-11). In order to minimize the effects of pseudo-replication [13], in each sampling interval, a pair of plots (one of the 3 treated and one of the 3 control) were randomly selected. Therefore, in each sampling interval, the traps contained in the selected plots (49 repetitions in the treated plot, and 49 in the control plot) were considered to perform the calculations.

Once in the laboratory, the traps of the pair of plots selected were temporarily stored in a cold chamber (4 °C), and then examined with a binocular magnifying glass for the taxonomic determination of the captured specimens and quantification. Among the species captured in the yellow sticky traps, special attention was focused on those species of major importance described as entomophagous associated with olive pests [7; 11].

2.3. Statistical analysis
For the statistical analysis of the data, the Statgraphics Centurion XVII (2016) and STATISTICA (13.0) statistical packages have been used. Firstly, the normality of the distribution of the catch data was verified by the Kolmogorov-Smirnov test (K-S, with the Liliefors correction). Since the normality tests indicated that the data set did not fit as a normal distribution, a square root transformation was applied in order to obtain a parametric data matrix to carry out the subsequent statistical analysis.

Once the normality of the data was accepted, the t-Student test was carried out. This test was made to compare the capture values of the beneficial insect in the treated and control plots. The cumulative calculation was used to visualize the variation of the relative error of the mean as a function of the sample size.

3. Results and Discussion
A total of 2838 individuals corresponding to 5 species of natural enemies have been captured. Table 1 represents the species in decreasing order of abundance, with their mean and standard deviation values.
The most abundant species has been, by far, the predator *Aeolothrips intermedius* (Bagnall, 1934) (Thys., Aeolothripidae), with 59.30% of the total catches. It is a very polyphagous species and very relevant in the control of phytophagous mites such as *Oxycenus maxwelli*, *Tegolophus hassani*, and *Tetranychus urticae*. In the olive grove, it is associated with the phytophagous thysanopter *Liothrips oleae* [14].

The rest of the captured natural enemies have been parasitoids. The most abundant species of parasitoid has been *Chelonus eleaphilus* (Silvestri, 1908) (Hym., Braconidae), with 15.12% of the total catches. This parasitoid is an ovolarvian endophage, of high fecundity (more than 500 eggs / female), and very useful in the control of *Prays oleae* (Bernard, 1788). The efficiency of this parasitoid is remarkable, especially during carpophagous generation, when the clutches are very grouped, reaching parasitism rates of 80% [15].

*Ageniaspis fuscicollis* (Dalman, 1820) (Hym., Encyrtidae), with 10.29% of total catches, has been one of the most abundant species of parasitoids. It is a very effective ovo-larval, poly-embryonic and parthenogenetic parasitoid that can reach parasitism rates of 70% [15].

*Elasmus flavellatus* (Fonscolombe, 1832) (Hym., Eulophidae) had 8.24% of the total catches. The species of this genus are mainly larve ectoparasitoids of lepidoptera (Coleophoridae, Gracillariidae, Cosmopterigidae, Gelechiidae, Platellidae, Psychidae, Pyralidae, Tortricidae, Praydidae) [16-19]. In the olive grove, it is one of the most significant species in the parasitic complex associated with *Prays oleae* [20], presenting a remarkable activity on the anthophagous and philophagous generations [21].

Finally, *Pnigalio mediterraneus* (Ferrierre & Delucchi, 1957) (Hym., Eulophidae) was the natural enemy with the fewest number of catches collected (7.04%). It is an ectoparasitoid that mainly attacks *Bactrocera oleae* larvae, as well as leaf-mining microlepidoptera, including the philophagous generation of *Prays oleae* and the graciliarid *Metriochroa latifoliella* (Milliere, 1886) [22].

### Table 1. List of the main beneficial insects species captured in the study.

| Species                  | Control | Treated |
|-------------------------|---------|---------|
| **Aeolothrips intermedius** | 27/09-04/10 428 8,73 6,55 | 04/10-11/10 260 5,31 3,07 |
| **Chelonus eleaphilus**   | 27/09-04/10 34 0,69 0,87 | 04/10-11/10 114 2,33 2,01 |
| **Ageniaspis fuscicollis** | 27/09-04/10 78 1,59 1,40 | 04/10-11/10 56 1,14 1,27 |
| **Elasmus flavellatus**   | 27/09-04/10 79 1,61 1,57 | 04/10-11/10 54 1,10 1,57 |
| **Pnigalio mediterraneus** | 27/09-04/10 56 1,14 1,38 | 04/10-11/10 43 0,88 1,05 |
3.1. Determination of the sample size based on the relative error of the mean
Due to its greater abundance, in this study, the predator *Aeolothrips intermedius* has been selected as the species on which the relationship between the sample size and the estimation error will be established. This relationship is represented in Figures 1 and 2, which correspond to the two sampling periods. For the first sampling interval (Figure 1), in the control plots, the relative error decreased to less than 10% from 17 repetitions, and from 13 repetitions in the treated plots. For the second sampling interval, the relative error decreases under the 10% to a number of repetitions greater than 16 and 9 in control and treated plots, respectively. It allows us to assume that a sample size of 17 repetitions ensures acceptable estimates, with an error of less than 10%.

Figure 1. Relative error of the mean capture value of the predator *Aeolothrips intermedius* (sampling 27 / 09-04 / 10) in relation to the increase in the sample size. The number of repetitions corresponding to an error of less than 10% is indicated (red color) for the control (○) and treated (▲) plots

Figure 2. Relative error of the mean capture value of the predator *Aeolothrips intermedius* (sampling 04/10-11/10) in relation to the increase in the sample size. The number of repetitions corresponding to an error of less than 10% is indicated (red color) for the control (○) and treated (▲) plots
The determination of the minimum number of repetitions to obtain acceptable estimates that allow determining significant differences in the statistical comparison tests between treated and control plots is 17 repetitions. This will allow future studies to adapt the plot size for the application of the proposed technique, in order of detection of truly organic crops, reducing as far as possible, the area of application of the experimental treatment, and therefore allowing obtain reliable results, interfering as little as possible in a fully organic crop.

3.2. Determination of the minimum sample size to detect statistically significant differences between treated and control plots

The results of the statistical comparison of the data series of the treated and control plots, corresponding to increasing sample sizes, are indicated in Table 2.

Table 2. Results of the t-Student test for an increasing number of repetitions (n = 3 to 40) in the capture data of *A. intermedius*, corresponding to the two sampling intervals. [p <0,05 (*); p <0,01 (**), p<0,001. (***)]
From the data from the first sampling, we observed that to obtain statistically significant differences (p < 0.05), a sample size of at least 6 repetitions would be required. By analysing the data from the second sampling interval, statistically significant differences between the treated and control plots can be detected with a minimum sample size of 17 repetitions (p<0.05). The highest level of statistical significance (p <0.001) was observed with a minimum sample size of 13 repetitions in the first sampling, and 21 repetitions, in the second.

In general, the results of the two analytical approaches studied here are relatively coincident, in that the sample size of 17 replications allows estimates with an acceptable margin of error, and at the same time allows the detection of statistically significant differences to determine the effect induced by insecticide application, on the behaviour of insects. This sample size suggests that to obtain reliable results, an area of approximately 100 m² would be needed for the experimental application of the insecticide. In relation to the mean surface of an average olive grove, usually greater than 10 Has, the area devoted to the application of this analytical technique represents a proportion less than 0.1% of the total crop, to be used for the induction of sublethal effects by the insecticide application. This suggests that the impact exerted at the agroecosystem level by this technique would be practically insignificant. Therefore, the use of this methodology is presented as a complementary tool for the verification of organic crops, and its application would not be an obstacle for truly organic crops to lose their certification.

4. Conclusions
This study made it possible to select the species *Aeolothips intermedius* as the most suitable beneficial insect to be considered as a bioindicator in studies intended for the verification of olive groves under the organic cultivation regime.

According to the objectives, the determination of statistically significant differences for the effective application of this methodology, based on the induction of sub-lethal effects in beneficial insects and the subsequent monitoring using a sticky yellow traps device, requires a minimum of 17 replications.

In line with the above, the results have allowed determining that the minimum size of the experimental target plot, to obtain acceptable population estimates (relative error of up to 10%), would require a minimum area of at least 100 m², representing a minimum number of 17 olive trees.

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