Influence of Pesticides on the Micro-Mycelia Present upon the Grape' Surface

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Abstract

The influence exerted by pesticides upon the microorganisms which are present within the vineyard' soil or upon the grape bacca' surface is a theme which has been approached by a lot of scientists. Our researches too come to join this line of investigation, focusing upon the effects exerted by some organically chloride pesticides on the micro-mycelia which are usually to be found upon the surface of the grapes' bacca. As a pesticide, we have made use of a product which relies on folpet as its active substance. Our experiment was carried on within a viticultural plantation and its aims were to study both the remanent effectiveness of the applied pesticide and the micro-organisms' subsistence after the concerned pesticide was applied. The stages of our experiment were: immediately after the applying of the pesticide (that is to say 24 hours since it was applied), respectively 7, 14 and 21 days consecutive to this moment. Thereby we have ascertained the fact that the applied product does act mostly upon the mildews but less upon the yeast' strains. We have also remarked that the concerned product does function at its greatest effectiveness immediately after being applied.

Keywords: grapes, folpet, mildews, pesticides

1. Introduction

The continuous development of the consumers’ expectations in regard to the respective amounts and qualities of the horticultural productions (fruits, vegetables, grapes and their respective consequently derived from products) has brought an increase for the number of existing horticultural exploitations of the intensive type; the implicit result of this fact is the use made of pesticides at a constantly increasing extent (this phenomenon goes for both fungicides and insecticides). Due to these circumstances, the individuals who do form the macro-fauna and the microflora of the concerned horticultural biological systems have come to develop some intrinsic mechanisms which do enable them to resist. Therefore it has become imperative to select the pesticides which could have, simultaneously: the quickest occurring effect; the longest lasting effect; the most precise pin-point effect. In the current practice of agriculture and of horticulture, a large amount of the applied pesticides do reach to the soil and remain within it; thus, most of the researches dedicated to the effect exerted by pesticides upon the microorganisms were orientated towards the soil’s microflora [4, 5, 7, 8, 9].

A relatively recent publication (2011) has brought to our attention the results of some researches carried on in Slovakia and concerning the consequences created by the action of adding pesticides upon the leaves and fruits' colonization with yeast' strains [10]. However, we are able to ascertain the fact that to the influence exerted by pesticides upon the useful micro flora (that is to say yeast' strains owning a high fermentation capacity), little attention has been granted yet at the worldwide level [1, 2, 6]. On the surfaces of grape baccas, the distribution
of the existing microorganisms is not at all uniform; this feature does as well concern their respective amounts and their taxonomy, so that some of them might be predominant in respect to the others. Should the pesticides take a preferential aiming action upon the useful microorganisms and should these latters happen to be less represented in the matter of their respective amounts, then the concerned substratum would come to be metabolized by other species, which would have proven to be more enduring. All microorganisms do, however, dispose, due to their inner biological and chemical systems, of a large diversity of enzymes which do own the capacity of deteriorating a part of the substances which can be found in the composition of pesticides; therefore, under apparently hostile circumstances, they still manage somehow to survive. Gallardo G. and all. [3] (2012) has demonstrated the fact that some among the existing microorganisms are able to deteriorate the pesticides' residues which might result, thereby preventing these latters' accumulation within the fermented final products. In our country, similar researches were performed by a collective of scientists from the Al. I. Cuza University in Iasi, in cooperation with the U.A.S.V.M. from Bucharest. The fact has also been proven that the products made use of through their applying in order to clarify the wine are as well contributing to the eliminating of the pesticides' residues which might have remained within the wine by “landing” there from the grape baccas' surfaces [11]. The present study comes in order to follow the above mentioned research line, namely the study of the pesticides' exerted influence upon the grapes' microflora.

2. Materials and Methods

The concerned experiment was conducted within a private viticultural plantation, upon the Cabernet Sauvignon kind. The active substance within the applied pesticide was the folpet. Figure 1 does present its chemical formula.

![Figure 1. Chemical formula of folpet](image)

The amounts of existing pesticide residues were determined after 7, 14, respectively 21 days. Simultaneously, the grapes' microflora was also evaluated. In order to determine the amounts of pesticide residues, the grape sample was prepared the following way: at first, the grapes were duly rendered of a homogeneous consistency; then, from the chosen sample an exact weight of 5 g was prelevated and placed within the tub of a centrifugal device, in order to be extracted. In the respective tub had been also previously placed 10 ml of acetone, 10 ml of dichloromethane and 10 ml of oil ether. After the mixture had been stirred up, it was centrifuged. Next, 18 ml of the obtained result were prelevated from the supernatant by means of a rotavapourizing balloon with a capacity of 25 ml; its contents was vaporized and the remaining residues were handled again through 3 ml of a 9:1 mixture of iso-octane-toluene. The balloon was, next, sonicated so that the pesticide residues could be more effectively recovered through the use of the solvent. From the resulting mixture, the residues were injected within a chromatographic gas. The employed device was an ECD Varian CP-3800CP; the chosen temperature programming’s were: from 50°C, timing grade of 1 min, increase by 15°C/min; from 200°C, timing grade of 0 min, increase till 235°C by 5°C min⁻¹; from 200°C,
timing grade of 0 min, increase till 290°C by 20°C min⁻¹. The witness sample made use of for comparison purposes on the occasion of the present study was represented by grapes which had not been sprinkled with the studied pesticide. The concerned vine log was duly isolated prior to the pesticide's applying and it was maintained under isolation conditions for 24 hours, so that its contact with the pernicious substance could be avoided. All samples were transported, in the briefest possible delays, to the specialized laboratories of micro-biology, respectively of residues and contaminating substances; the analyses upon them were performed within a time lapse of less than 6 hours since their harvesting. In order to determine the respective amounts and qualities of the micro-mycelia that were present upon the grape' surface, an YMA fungi-profiled environment was made use of (that is to say malt extract - agar - yeasts' extract). The incubation period was of three days, spent at 25°C; the first observations were performed at 24 hours since the insemination. The numbers of resulted colonies were duly recorded. To this purpose, the colonies' counter with which our laboratory is endowed was made use of. The quality analysis meant the identifying of the microorganisms' types, in regard to the ones which had generated the colonies that we have spotted upon the surface of the hosting environment.

3. Results and discussion

The presence and the distribution of micro-mycelia (that is to say yeast' strains and mildews) upon the surface of grape baccas have been since a long time studied by specialists, who have reached to the unanimous recognition of the fact that these microorganisms are not uniformly disseminated, neither in the matter of their respective amounts or in the one of their taxonomy. The action exerted by pesticides upon the micro-mycelia might be thought of as being aimed in a way that ought to make it as precisely targeted as possible, because it is a known fact that some among these microorganisms are, indeed, able to deteriorate the pesticides' active substances due to the enzymes they are endowed with. In the horticultural practice, the action of applying pesticides is carefully placed under legal dispositions meant to rule it. Thereby, their sought purpose is the one of; as much as it could be feasible, diminish the pesticides' remanence duration. Thus, the European Union's regulations do establish, in this respect, certain highest admissible values for the residues lasting from pesticides and/or other contaminating substances. Upon the surface of the grapes destined to the wine-making process, the European Union’s legislation has chosen to admit a highest limit for the tolerated amount of folpet, a limit which is of precisely 10 mg/kg.

Determining of concentration for the folpet's amount which might persist upon the grape bacca' surfaces

The folpet is known and made use of as an active substance which usually functions as a part of the chemical composition of some organically chloride pesticides applied in the viticultural practice. Its chemical formula is presented in Figure 1. This fungicide does inhibit the microorganisms' normal cellular division; consequently, the ones which do not dispose, in their own enzymes’ equipment, of the appropriate biological catalysts able to destroy the concerned residues co come to be annihilated, even at low folpet concentrations. Due to the fact that the toxic action they might exert upon the human organism would be highly acute, the amounts of folpet residues which might persist upon the grape bacca'surfaces are very attentively monitored. On the ground of the results recorded after 7, 14 and, respectively, 21 days since the pesticide was applied, our observations have ascertained of an almost linear reducing of the concerned concentration. Figure 2 does present the etalon of folpet. The obtained results do show the fact that, from an initial concentration of 2, 5 mg kg⁻¹, after only one week, it decreases to 1,02 mg kg⁻¹. Figure 3 present the chromatic diagram realized...
7 days after the treatment’s moment. After 14 days, the folpet's concentration does appear as reduced to half of the initial level (Figure 4), that is to say it becomes precisely 0.515 mg kg\(^{-1}\); after 21 days, only 0.238 mg kg\(^{-1}\) of folpet do subsist upon the grape’s surface (Figure 5).

**Folpet's effect upon the micro-mycelia which are present upon the grapes bacca' surfaces**

In order to determine what might be the effect of folpet upon the micro-mycelia which are present upon the grapes bacca' surfaces, some residues were prelevated straightly from the grapes bacca' surfaces and inseminated within an YMA culture environment. A sanitation swab was employed to this purpose. Parallel experiments were conducted upon some grapes treated with folpet as well as upon some grapes not treated at all.

The untreated vine logs were carefully isolated for the duration of the whole sprinkling operations' process, so that they could in no way enter in contact with the concerned fungicide. The inseminations were accomplished during the same days when the amounts of folpet residues were determined, yet with the mention that the first insemination was done 24 hours after the product had been applied. After the operations of insemination and of thermostatic conditioning, the concerned plates were studied in order to record both quantitative dimensions and qualitative features (pertaining to taxonomy matters). In the images below (Photo 1 and 2) do present colonies which have developed upon the culture environment after the moment when the grape' samples were inseminated, that is to say 24 hours after the fungicide had been applied.
As we are able to notice in the Image 1, from the surfaces of the untreated grapes the isolated types were, mainly, mildews (colonies of which the colors were situated in a range from grey to black) and pellicle-shaped yeast' strains (large colonies bearing colors going from white to pale grey). On the ground of the performed morphological tests, some facts have been ascertained: - the mildew which has developed colonies of colors going from grey to black with a reverse side which is black only and provides an impression of fluffy when touched has belonged to the *Aspergillus* type. Frequently found as disseminated within the soil or on the respective surfaces of fruits, of vegetables or of seeds, the *Aspergillus* type does indeed include some species which do act as toxins towards mycelia. Furthermore, through its quick spreading, it also might cause alterations for large quantities among the harvested grapes. On the ground of the performed morphological and physiological tests, the facts were also proven that, for the cases of the white till grey colonies which presented smaller dimensions, they had been developed by species of the *Pichia* kind; in what concerns the giant colony, we are speaking of a species pertaining to the Candida type. In the case of grapes treated with folpet, our observations did ascertain the fact that, in what concerns their quantity, the fungi had significantly diminished (Photo2). The pellicle-shaped yeast' strains which had been spotted at the untreated sample were not at all affected by the fungicide's presence, developing them almost identically as in that former case. Furthermore, in the latter situation we have also isolated and ascertained the presence of some other types of yeast' strains (small colonies of a creamy white color). The performed morphological and physiological tests have allowed us to frame them within the *Kloeckera* type, about which it is a known fact that it does usually populate the grape bacca'surfaces in a percentage ratio possibly rising till around 90% of the total amounts of the present yeast'strains. Their remarked absence from the bacca' surfaces of the untreated samples might be explained through the fact that it is the mildews which had, very quickly, taken the natural culture environment’s possession (that is to say of the grapes bacca' surfaces), thereby inhibiting the other components of the micro flora. As for the rest, there are studies which have demonstrated that, in the presence of folpet, *Kloeckeraapiculata* may be stimulated to produce more alcohol. We are also due to mention the fact that our collective had chosen to carry on its researches under climate conditions which were favorable to the development of mildews (an excessive humidity of the atmosphere, brought by the abundant amount of precipitations from the grapes' maturation period). Along with the mildew which belongs to the *Aspergillus* type, another two types have found suitable conditions to develop themselves: the *Penicillium* (a greyish green colony with a white border) and the *Alternaria* (a large and fluffy colony, the color of which was a dirty greyish white).
Consequently, the applied treatment involving the folpet had as its immediate result the considerable diminishing of the mildews’ amount in regard to the *Aspergillus* type. Seven days after the fungicide was applied, when the ascertained amount of residues was noticed as being of 1, 02 mg kg\(^{-1}\), the fact has also been remarked that, in the case of the untreated sample, a high number of mildew colonies had developed (Photo 3).

For the same sample, the yeast’ strains percentage was smaller than the one of the previously mentioned sample; this fact could only be explained through the existing conditions involving an excessive humidity, which had acted as a stimulus for the mildews. We have also ascertained the fact that, apart from the type-casted colonies coloured in a darkening grey which are the distinctive indication for the presence of *Aspergillus niger*, another *Aspergillus species* had also developed, namely the *Aspergillus flavus*, which does form colonies of an yellowish white color, which are flat and velvet-like; some colonies belonging to the *Penicillium* type had appeared too. Since the mildews had developed that much, the yeast’ strains were, thereby, considerably less represented. We have to mention the fact that, at the microscopic level, no sign of whatever mildews’contagion was ever spotted as being present within the grapes, because, for the purposes of isolation and insemination, we have deliberately sought for the healthiest among baccas. At the grape’ sample treated with folpet, 7 days after the treatment had been applied, we have ascertained some slight modifications from the initial status, in the sense that, as long as most of the mildews had come to be effectively inhibited, the pellicle-shaped yeast’ strains had, consequently, also been allowed to develop themselves very well (Photo 4). The third stage of our investigation has taken place at 14 days since the treatment's moment, when the pesticide residues’ amount had come to be reduced to its half in comparison with the previous stage of our research (to 0,515 mg kg\(^{-1}\)). From the microbiological perspective, the fact we have ascertained is that, upon the surfaces of the untreated grape' baccas, a great amount of mildews does keep on developing (Photo 5), most of which do belong to the species of *Aspergillus flavus* (yellow colonies), of *Penicillium* (the colonies' colour is green with their borders white) and of *Alternaria* (large, white and “fluffy” colonies). Finding themselves submitted to this occurring phase in the mildews ‘development, the yeast’ strains were almost completely inhibited. The supervened changes in the architecture of the mildews might be explained through some corresponding modifications which had appeared in what concerns the climate conditions. After 14 days since the treatment had been applied, the facts we are able to ascertain are, on one side, that, in the matter of taxonomy, the mildews are approximately of the same types for the two samples and, on the other side, that the differences between the sample treated with folpet and the untreated one do mainly appear in what
concerns the yeast’ strains, which have better developed within the folpet-treated sample by comparison with the untreated one (Photo 6).

![Photo 5. Colonies which have developed upon the culture environment (sample of grapes untreated)](image1)

![Photo 6. Colonies which have developed upon the culture environment (sample of grapes treated with folpet – after 14 days)](image2)

The decrease of the remanent effect exerted by the applied fungicide was the most visible at 21 days since the treatment had been applied, when the pesticide residues’ amount had decreased to the level of only 0.238 mg kg\(^{-1}\). We have to remark that, at this moment, between the two samples the existing differences do pertain rather to quantity than to quality, in the sense that the same types of mildews do develop within both samples, but, within the untreated sample, the respective amounts from each involved type are increasing (Photo 7) when compared with the ones held by the treated one (Photo 8). Therefore, we may notice that, in the untreated sample, the species of *Aspergillus niger* is predominant, while in the treated sample we have ascertained an existing equilibrium among the respective species of *Aspergillus, Alternaria* and *Penicillium*.

![Photo 7. Colonies which have developed upon the culture environment (sample of grapes untreated)](image3)

![Photo 8. Colonies which have developed upon the culture environment (sample of grapes treated with folpet – after 21 days)](image4)

4. Conclusion

In the horticultural exploitations of the intensive type, the applying of pesticides is unavoidable yet bearing all the consequences which this fact does indeed suppose; among them are included the effects exerted upon the microorganisms which are usually present within the soil, on the leaves or upon the respective surfaces of fruits and vegetables. The pesticide’ residues which might be able to subsist upon the respective surfaces of fruits and vegetables ought to be attentively monitored, since it is a known fact that they all do cause various but highly toxic effects upon the human organism. The fact has been ascertained that...
the folpet substance, which is an usual component of various fungicides applied in the common practice of viticulture, does considerably reduce its amount at 21 days after being applied; consequently, the subsisting (and respectively measured through scientific methods) quantities of pesticide residues do come to be, indeed, much smaller than the maximum limit which is legally allowed by the European Union's regulations. The facts have been ascertained that the “folpet” does act the most effectively, among all the microorganisms which are usually present on the surfaces of grape baccas, upon mildews, especially upon the ones from the *Aspergillus* type, but also that its action does persist for only the former 20 days since the concerned treatment is applied and that, after this important moment, the “folpet”s effect does considerably diminish. The high concentrations of the applied folpet almost did not affect at all the respectively present yeast’ strains in their generality (and this fact was especially proven about the pellicle-shaped ones); thereby, even after an interval of 24 hours since the concerned fungicide was applied, the respectively present yeast’ strains have continued to develop themselves in their normal way.

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References