Biological responses to advanced foliar fluidic nutrients applications

CAMELIA CRISTEA*, GHEORGHE CAMPEANU, LAURENTIU FILIPESCU*, MARIANA STEFAN*****
** Faculty of Industrial Chemistry, University POLITEHNICA of Bucharest*
***Faculty of Biotechnology, Bd. Marasti No. 59, 71331, Bucharest, Romania*
****Industrially School Group "Nicolae Teclu" Bucharest, sect 3, str. Ion Tuculescu, No. 42.*

Abstract

The new approach on emulsified nutritive fluids formulation opens a path to graft multifunctional properties such as growth enhancing and fungi repelling to common foliar fertilizes. Selection of overbasic potassium salts of naphthenic and oleic acids as organic phase carrier in these formulations has been grounded on their proven antifungal attributes. In this paper, the diffusion method of antimicrobial susceptibility test was adapted to quantify antifungal properties of the potassium overbasic naphthenates and oleates as intermediary products in the manufacture of nutritive fluids. The results have validated the proficient fungicide effects of potassium overbasic naphthenate with overbasicity 4/1 and the mild behavior of potassium overbasic oleate as contact fungicide.

Keywords: foliar fluids, fertilizers, biostimulants, fungicide

Introduction

It is beyond any doubt that one the most simple and efficient way to handle crop control during any vegetative development stage resumes to the use of meaningful formulations of foliar fluids like fertilizers, growth enhancers and pesticides. A short review over the properties of these foliar fluids (complete solubility in hard waters, hiding power, adherence, penetration power through waxy cuticles, moderate pH, low saline and contact stress, environmental friendliness, etc.) shows that a great deal of them could not be associated with saline solutions of nutritive metal compounds or with mixtures of growth control-pesticide organic substances.
Our previous research brought about a new approach to the subject, apprehending the foliar penetration mechanism and its kinetics as a keystone in formulating foliar fluids and product properties modeling [1-9]. According to this concept, these foliar fluids have to be concentrated emulsions containing two distinctive stages: the organic stage which is the carrier of functional growth enhancing and fungicide compounds and the aqueous carried stage which is conveying the mineral burden in terms of NPK plus micronutrients formula. The original approach of this formulation is visualized by the following worthy features: a) The concentrated emulsion contain reactive components which are able to release after dilution and application new born biological active entities; b) The organic stage is a bioactive component capable to penetrate the cuticular barriers of leaves and it may carry additional growth enhancing or fungal functions; c) At the foliage level the diluted fluid leaves an organic matrix made up by hydrolysates of the organic phase able to maintain a minimal humidity on foliage surface; d) All new born biological active entities are released by organic matrix as nanoparticulate amorphous matter, as well as micelles charged with amorphous nanoparticulate matter; e) All nutritive fluid components are biodegradable and environmentally harmless. Organic phase selection criteria and properties control of

intermediary organic products bearing growth enhancing and fungal functionality have recently been studied [10]. It was found that potassium overbasic naphthenates and oleates were properly gathering all the attributes required for organic phase in terms of compatibility with the aqueous stage, reactivity with air free carbon dioxide, hydrolysis pH, hydrolisates particle size distribution and may carry growth enhancing capacity [2,4,9,10]. This paper deals with the fungal functionality of potassium overbasic naphthenates and oleates which might be handled to shore up multiple biological effects to the new class of nutritive foliar products. The aim of this investigation is to learn more about each particular intermediary product and to develop both proper manufacture and application technologies. To this extent, the diffusion method of antimicrobial susceptibility test was adapted to quantify antifungal properties of the potassium overbasic naphthenates and oleates at concentrations close to those applied in the field. Basically, antifungal impregnated paper disks are stuck firmly on the surface of a fungi culture appropriate medium that has been previously inoculated with a pure fungi stains suspension. After incubation, zones of inhibition are measured and correlated for assessing the minimum inhibitory concentration (MIC) [11].

Materials and Methods

Intermediary product samples preparation. Sample of potassium overbasic naphthenate and respectively potassium overbasic oleate with overbasicity (molar ratio K_2O/organic acid) 4/1, as intermediary products in manufacture of foliar nutritive fluids, were carefully prepared from purified reactant at 1 mol KOH/liter concentrations. These solutions will be further ranked as active fungicide components in foliar fluids and refer as basic solutes when samples were diluted for the trials designed to evaluate minimum inhibitory concentration (MIC) of each intermediary product.

Inocula preparation. A standard young strain of *Alternaria spp* and respectively *Botrytis spp* was selected to test antifungal properties of the above intermediary products. Each selected culture was spread onto a non inhibitory agar medium to obtain isolated colonies. After a few days of incubation, well-isolated colonies were subtracted before sporulation or conidia growth to avoid the outset vegetative forms which are more sensitive and do not yield confident results in such trials. Specimens of these inocula were suspended in sterilized water, shaken for homogenization until an opalescent fluid is substantiated and additionally left for 10 minutes for air separation. The fungal suspension should then be compared to the 0.5 McFarland standard. Consequently, the opalescent fluid is poured into small drops under continuous mixing into a tube containing 3 milliliters of sterilized water and some glass beads until the sample reach the opalescence of the 0.5 McFarland turbidity standard corresponding to $0.4x10^4$- $5x10^5$ CFU/ml. Thus, the inocula standard samples of *Alternaria spp* and respectively *Botrytis spp* are ready for application.

A fresh McFarland 0.5 standard has to be previously prepared under tight quality control and stored in the dark vials for further experiments.

Experimental procedure. Intermediary products (potassium overbasic naphthenate and respectively potassium overbasic oleate) samples were diluted to a row of concentrations in a plausible range of field applications on foliage: 10, 20, 30, 40, 50, 50, 60, 70, 80 and respectively 90mg/l. These concentrations are taken as available doses whose antifungal power has to be tested. Actually, the antifungal solutions with each of the above concentration are impregnated on sterilized Whatman paper disks with 6 mm diameter. Each disk is moistened with 100 µl of diluted samples in the row of above concentrations, dried off under mild conditions and deposited in sterilized container. When the experiment was expended over more days the container was stored in the refrigerator (4°C) and subsequently left unopened for 1 hour at room temperature to avoid water of condensation on the disks. Sabouraud media freshly prepared was cast on Petri dishes and quickly inoculated each of

them with approximate 250 µl of each one of inocula containing $0.4x10^4$- $5x10^5$ CFU/ml. The liquid is spread over entire surface of plate and left for 3-5 minute, but no more than 15 minutes for full penetration through Sabouraud media, according to a standard procedure [11]. Next, the paper disks carrying the antifungal doses are repartitioned on the inoculated Sabouraud media surface, holding distances of 30mm between centers of two neighborly disks and 15mm from the borders of Petri dish. Thus, 4 or 5 disks were placed on each 100-mm plate. This procedure prevented measurement errors due to the overextended zones of inhibition. The impregnated disks are carefully pressed onto the surface of the culture media to back up reliable contacts. Because the antifungal product diffusion from the disk growth media commences moments after the application the disks were firmly bounced in a single stroke. All Petri plates prepared according the above procedures are incubated for 72 hours at 25º. If the antifungal product is active and the dosage was correctly chosen, it has to diffuse radially through inoculated media blocking all inocula and leaving behind clear spots free of fungi infestation. After full incubation, the zone of complete inhibition diameter was measured on all the plates without opening the lid and recorded it in millimeters. The minimum inhibitory concentration (MIC) is proven to be the concentration value at which the smallest diameter of complete inhibition zone was observed. Other considerations may be assigned in accordance with the missing zone of complete inhibition, vegetative growth and fungi survival inside the zones of complete inhibition.

Experimental development. Antifungal susceptibility test was performed for 2 fungi varieties *Alternaria spp*. and *Botrytis spp.,* in one replicate for *Alternaria spp.,* and two replicates for *Botrytis spp.* The experiment antifungal products were 5 samples containing the following components: A) potassium overbasic naphthenate (PON); B) potassium overbasic oleate (POO); C) mixture 1/1 volume PON +POO; D) mixture 3/1 volume PON +POO; E) mixture 1/3 volume PON +POO, prepared as was described above. Minimum inhibitory concentration (MIC) was evaluated from data collected on testing the following sample concentrations in all 3 replicates: 10, 20, 30, 40, 50, 50, 60, 70, 80 and respectively 90mg/l for all 5 samples. Temperature of incubation was 25-30ºC and duration of incubation was 48-72 hours. Additionally, photographs were taken for all sample plates with HP945 camera using adequate software for picture processing.

Results and Discussion

The first test on *Alternaria spp*. has shown that intermediary products, potassium overbasic naphthenate and potassium overbasic oleate, are bearing antifungal properties and could certainly prevent *Alternaria spp*. survival and growth starting from a concentration of 50mg/l in the applied fluids. Even if the antifungal activity is outright at 10 mg/l and rises unambiguously with the product concentration (table 1, figure 1), the minimum inhibitory concentration has to be placed at higher concentrations, where the clear spot diameter is around 20 mm (figure 2). Because both products are contact fungicides and their activity is sustained only inside the covered area, we have to assent that a minimal 100 mg/l of each product applied in the field can certainly inactivate *Alternaria spp.* Surprisingly, the mixtures of both intermediary products stand up active only for ratios higher than 3/1, where perhaps POK balances the antifungal performances. Thus, in this case the MIC concentration may be taken also for 100 mg/l. At other smaller ratios, the mixtures do not eradicate *Alternaria spp*., but can prevent sporulation and hold only on vegetative growth.

Table 1. Experimental data for the determination of minimum inhibitory concentration

Product concentration, mg/l	*Alternaria spp*				
	POK	POO	POK/POO 1/1	POK/POO 3/1	POK/POO 1/3
10	6	8	N. S. + C. V.	0	N. S. + V. G.

20	-	-	N. S. + V. G.	-	N. S. + V. G.
30	8	10	N. S. + V. G.	6	N. S. + V. G.
40	-	-	N. S. + V. G.	-	N. S. + V. G.
50	10	12	N. S. + V. G.	8	N. S. + V. G.
60	-	-	N. S. + V. G.	-	N. S. + V. G.
70	16	16	N. S. + V. G.	10	N. S. + V. G.
80	-	-	N. S. + V. G.	-	N. S. + V. G.
90	22	20	N. S. + V. G.	12	N. S. + V. G.
Product concentration, mg/l	*Botrytis spp* I				
10	14	8	-	8	N. S. + V. G.
20	-	-	-	-	N. S. + V. G.
30	18	10	8	10	N. S. + V. G.
40	-	-	-	-	N. S. + V. G.
50	20	12	10	12	N. S. + V. G.
60	-	-	-	-	N. S. + V. G.
70	11	14	10	14	N. S. + V. G.
80	-	-	-	-	N. S. + V. G.
90	26	14	10	18	N. S. + V. G.
Product concentration, mg/l	*Botrytis spp* II				
10	20	3	5	3	N. S. + V. G.
20	20	0	0	2	N. S. + V. G.
30	30	5	6	4	N. S. + V. G.
40	30	0	0	3	N. S. + V. G.
50	30	5	6	4	N. S. + V. G.
60	34	0	0	5	6
70	34	7	6	4	6
80	40	0	0	5	6
90	44	8	6	5	7
N.S. – no sporulation; V. G. – vegetative growth					

For these samples on all assay plates, the inhibition zones are missing (figure 2). We assume that each of the two products acts on its own mechanism over fungi crack down. These mechanisms do not overlap or interfere by any means in any stage of striking down the fungi. Thus, the POK and POO should be taken as two components acting on the same pest, but on different ways. Also, since the POO is a mild fungicide its real minimum inhibitory concentration should be thrust much more over 100mg/l.

Botrytis spp. seems to be second rated when competing with *Alternaria spp*., at least if POK is used as fungicide (table 1, figures 3 and 4). Its minimum apparent inhibitory concentration is somewhere around 10 mg/l. However, from the same considerations concerning the clear zones of inhibition (figures 3 and 4) and the contact sustained mechanism of inhibition, the MIC in the field conditions should be also displaced to 100 mg/l.

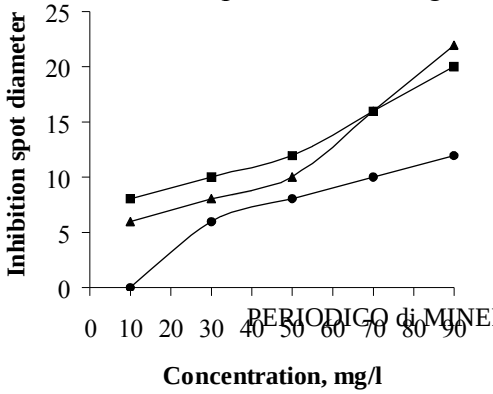


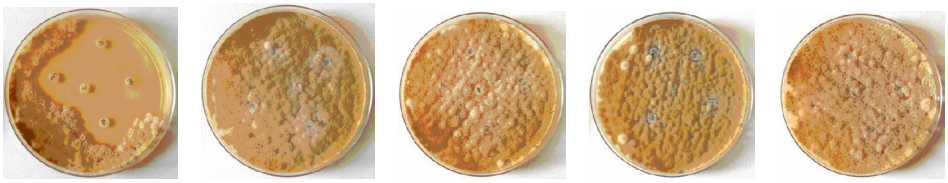
Figure 1. Intermediary products diffusion spot diameter. *Alternaria spp*. ▲K Naphthenate, ▪ K Oleate, ●K Naphthenate: K Oleate, volume 50:50

A B C D E

Figure 2. Pictorial diffusion spot. *Alternaria spp*.

A B C D E

Figure 3. Pictorial diffusion spot. *Botrytis spp.* I



A B C D E

Figure 4. Pictorial diffusion spot. *Botrytis spp.* II

Potassium ovebasic oleate has the same poor effect on *Botrytis spp.* as it has on *Alternaria spp.*. Even if some small zones of inhibition may be observed on figures 3 and 4, its antifungal activity is obviously not trustful. More over, the results of both tests might be concluded with the presumption that in the range of experimental concentrations the potassium overbasic oleate can only cease *Botrytis spp.* sporulation as far as vegetative growth is promoted. May be through a significant raise in concentration the product might gain a weighty antifungal activity. Because the pH of POO and POK-POO mixture solutions are higher than the pH of POK solutions [10] the theory of alkalinity sustaining antifungal properties of potassium compounds like carbonates or hydrogen carbonates seems to be denied.

Conclusions

The biological response of the advanced foliar nutritive fluids was investigated at the level of fungicide activity, using the diffusion method of antifungal susceptibility test as validation method. Two intermediary products – potassium overbasic naphthenate and potassium overbasic oleate with the overbasicity 4/1 were trialed to find out the minimum inhibitory concentration. Meaningful results were drawn over the antifungal power of each intermediary and the adequate concentrations to be applied on the field for a confident antifungal effect. The results have validated the proficient fungicide effects of potassium overbasic naphthenate and the mild behavior of potassium overbasic oleate as contact fungicide. The acting

mechanism of both intermediary products does not overlap or interfere by any means and the theory of alkalinity sustaining antifungal properties seems to be denied.

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