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Review Article

Development of Agromicrobicide Utilizing Synergic Joint Action of Active Ingredients

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Abstract

Enhancing pesticide action through mixtures is a powerful tool for improvement of current pest control technologies. In recent paper, various models of demonstration evincing synergic joint action are compared. The approaches to establishing experimental proofs to the satisfaction of Patent Offices and Patent Courts are discussed using the example of development of optimized mixtures of nitrofurane and benzimidazole derivatives. The antimicrobial activity of these mixtures surpass benomyl against fungi (Botrytis, Bipolaris, Fusarim and Ustilago) and streptomycin against bacteria (Erwinia and Xanthomonas). The optimized mixtures efficiently inhibit streptomycin tolerant bacteria and benomyl tolerant fungi.

Introduction

The organisms that might be deleterious under circumstances to man or cultivated areas can be declared to be a pest [1]. Multitudinous techniques have been developed and applied in human practices to combat losses of various goods used in everyday life, among them, the use of chemical substances – antimicrobial pesticides – to control pathogenic microbial species in croplands. The challenge is great as about 50000 microbial species threaten cultivated plants worldwide [2]. Recently, in European Union 1492 molecules exhibiting approved pesticide activity have been registered, among them 508 antifungal and 135 antibacterial ones, and 47 with dual effect, i.e., ingredients acting against both bacteria and fungi [3]. Probably, the same assortment is available elsewhere too, and about one fifth of the marketed worldwide of more than 2 million tons of pesticides is used to control phytopathogenic microbes [4,5], indicating the robust growth of utilization synthetic chemicals in agriculture which often induce calamitous effects on both environment and food chain.

Dramatic advancement in biology within the last fifty years opened through genetic engineering and gene technology to plant grow cells in suspension cultures with subsequent ability to regenerate whole plants that created a whole new era in plant breeding. Some efforts have been made, and there is an increasing interest to introduce alien genes coding performed defense molecules into cultivated plants. The experiences are contradictory: Unexpected adverse effect has manifested both in biocoenoses and in pests themselves, mainly due to acquired tolerance in populations of target organisms, like Lepidopteras to thuringiensis toxins or innumerable weed species to herbicides. No doubt about that microbes of agricultural interests will also rapidly adapt to new properties. Nevertheless, the intentions to improve the plant resistance by rationally designed genetic manipulations using biotech methods are promising together with to develop botanical preparations to combat losses in agriculture. However, some questions need answers, first of all problems of unwanted exposures. In spite of intensive studies on defense molecules, - such as phytoalexins and phyoanticipins, - our knowledge regarding their mode of action and the flow of signal transmitters from the pathogen to the plant cells is still poor. Nevertheless, the intense search for natural and safe alternative pesticides in recent years may help to find new, effective antimicrobial agents with novel modes of actions, i.e. promising candidates for lead compounds [6]. Also, there is an urgent need to investigate the mode of interaction synthetis pesticides and natural defense molecules, which is a white field of agronomic sciences.

Although the application of chemical pesticides is still preferred the most over all other alternatives to protect crops from yield loss, new challenges have aroused. Until intensification of the world market of seeds and other propagation materials in the past century some diseases remaining sporadic and threateting only croplands of formerly isolated or abandoned regions nowadays easily quit and spread worldwide. Moreover, decreasing biodiversity of croplands due to increasing mono-cultivation and also decreasing heterogeneity of varietal sortiments of cultivars favor the rapid distribution of phytopathogens. The cases of fire blight of rosaceous plants or downy mildew of cereals are well known examples. Although bioengineering some pest-resistant crop varieties using transgenic approaches to avoid pesticide use raised hopes [2], the application of chemicals is still preferred the most over all other alternatives to quarantee the yield sureness. However, the calculability of pest management applying synthetic monosite inhibitors has been threatened by rapid emergence of acquired tolerance to this group of chemicals. This situation urges new developments, search for new lead compounds as well as profound comparative studies of the physiology and genetics of pathogens and host plants to discover new target sites for selective inhibitors [6]. The knowledge both on mode of action of molecules with pesticidal activity and on their fate in the environment should be expanded, which is the basis of improvements of pest management practices [2].

Enhancing pesticide action through mixtures is a powerful tool for improvement of current pest control technologies. Numerous models have been constructed for evaluation of the character of joint action of chemicals [7], which mixtures meet special problems of assessment and data treatment. Joint action of compounds Z and V can be performed by different manner:

a) Additive or aggregative effect; the total effect is a simple sum of the proper effects of parties [PEZ and PEV], i.e.

$$[Z + V] \rightarrow W$$

where the expected effect [EEW] of W is equipotent to the measured effect (ME) of their mixture. In this case |EEW-ME| < $LSD_{0.05}$ of the analysis, and one can assume that the biological effects of the parties (Z and V) do not interfere.

b) Antagonism (counteraction) - opposition in biological action, i.e., interaction of two or more substances such that the action of any one of them on organisms is lessened, that means,

$$[Z+V] \rightarrow W$$

where EEW < ME, and |EEW-ME| > LSD0.05.

c) Synergism - interaction of compounds such that the total effect is greater than the sum of the individual effects, i.e.,

$$[Z+V] \rightarrow W$$

where EEW > ME, and |EEW-ME| > $LSD_{0.05}$.

In general, all experiments should be made as major care as possible, and changes in effectivity must be significant. The accepted level of significance depends on the experimental model applied for measurements; the acceptable significant difference (LSD) should be weighted in pure cultures at P<5%, working with plants or separated organs at P=5-10%, while in field experiments depending on circumstances the level must be P<15%.

Two basic standpoints should be considered in decision process of product development when patents support monopoly rights:

a) The novelty of a finding, which is an absolute criterion, however, there is relatively easy to find nowadays an earlier publication canceling the patent application.

b) The obviousness, which is a complex problem, and it relates to a number of headings; inventive level, inventive step and technical advance.

The first two aspects meet very subjective criteria. What seems to be masterfully inventive to one person can appear evidently obvious to another. However, the technical advance can be supported by experiments as well as by comparative analysis of the present level of technology. In present paper development of agro-microbicide of broad spectrum activity based on binary mixture of furazolidone and carbendazim (Figure 1) is demonstrated.

The optimized mixtures of nitrofurane and benzimidazole derivatives efficiently inhibit a large number of microbial species, particularly streptomycin tolerant bacteria and benomyl tolerant fungi [8]. Furazolidone, - formerly used in medicine and veterinary possesses antibacterial [9-11] and antiprotozoal [12] activities , - significantly and selectively inhibits (MIC < 25 mg/L) phytopathogenic Erwinia and Xantomonas [8], while carbendazim is a well known agricultural fungicide of systemic activity. The activity of furazolidone was first detected by agar diffusion technique testing activity against a strain of F. oxysporum tolerant to benomyl, and the synergic joint action with carbendazim was later verified against other *Fusarium* species as well (Figure 1). On the example of these molecules we demonstrate the steps of decision process (details of toxicological methods are published in cited papers [13,14]):

a) Demonstration of the synergy by agar diffusion technique.

b) Comparative analysis of dose/response lines.

c) Optimization of the mixture applying the model of *Horsfall and Dimmond* [15] for determination of the ideal mixing ratio as well as the Sun's model for calculation of the rate of synergy.

d) Characterization of the spectrum of activity of new preparation.

Results and Discussion

Agar diffusion technique

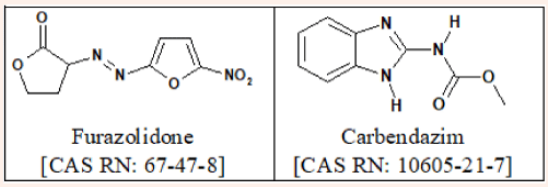

Figure 1: Structure of synergically interacting compounds.

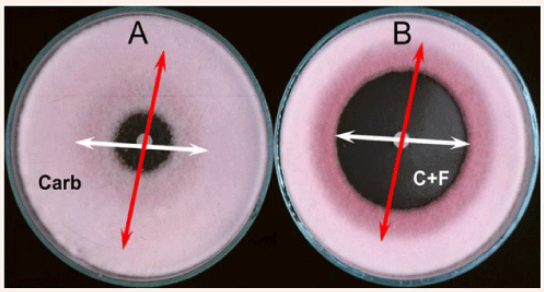

Figure 2: Joint action of furazolidone and carbendazim against conidium germination and hyphal growth of *Fusarium avenaceum* in axenial culture.

This is a rapid method useful for high throughput screening, however, due to its semi-quantitative character results obtained by this manner allow only crude approach of the phenomenon examined. First, extremely difficult to standardize the process, as the circumstances essentially influence the interaction between test organism and test substance, moreover, the physiology of test fungi changes throughout the exposure.

The agar diffusion technique - filter paper discs (5mm diameter) impregnated with 10.6 M of test compound were placed centrally on the agar plate (one disc per dish of 90 mm Ø) and diameter of growth inhibition zone was measured after 48 hours incubation at 22±1 °C. Details of the method other than here were published earlier [16].

Plate A - disc contained 10.6 M carbendazim.

Plate B - disc contained 10.6 M carbendazim an 10.6 M furazolidone. These results show evident synergy, but are not supportive for patent claims.

In the given example, carbendazim per se influences two ontogenetic stages of the fungus, namely, the conidium germination, – this was inhibited in transparent area of the plate, - and the hyphal growth (depressed growth of hyphae marked with white arrow), as well as intensified the red pigmentation arouses in affected hyphae (marked with red arrow); these two items are clearly concentration dependent phenomena (Figure 2). Around furazolidone no clear zone formed, thus the inhibition of conidium germination could not be observed by this method, and the mycial met was inhibited slightly over the paper disc, so its effect alone could be rated as insignificant (the plate is not shown). However, adding furazolidone to carbendazim, the zone of full inhibition dramatically increased (white arrow), and the physiological effect turned to be more pronounced (Figure 2). Seemingly, the activity of carbendazim was toned up, as the growth of fungus stopped in the area where its growth was only depressed without furazolidone (the size of these zones are nearly equal in two plates!), while the response manifested in pigmentation did not changed by means of the size of red arrows, although its concentration limit became more evident.

Optimizing the mixture for practical use

The applied ratio of two components in the above experiment was 1:1, and the synergy was convincingly demonstrated. To determine the optimum ratio a more accurate experimental model was taken out to obtain proofs of the phenomena which persuade the Examiner of Patent Office and support claims that stand requirements according to the decision of Patent Court in re Lemin's case [17]. Based on former experiences [18] the model proposed by Horsfall and Dimmond was used up with minor modification. The aim of examination of this mixture was to develop an antimicrobial preparate against seed borne bacterioses with simultaneous control of some pathogenic fungi that constrain production of seeds and other propagation materials. Antimicrobial activity was determined by the traditional poisoned agar gel method using the preparates at the concentration of 10 mg/L. After incubation the colony diameters were determined and the growth inhibition was calculated as percentage as related to colony growth on microbicide free media, details other than here were publisher earlier [14].

Abbreviations: SI - range of synergy for fungicidal (F) and bactericidal (B) activities, respectively. Hexp - expected fungicidal activities and Se – expected bactericidal activity, according to Horsfall's model. MPT - Most potent treatment, MRV - Maximum response value of X. stewartii (opened circle) to furazolidone. Bcmax and Bzmax are maximum response values of *B. cinerea* (diamonds) and B. zeicola (squares) to carbendazim, arrow S marks the difference between activity of optimized mixture and present state of technology (streptomycin), while the arrow C+F marks the overlapping interval of

mixing ratios where these most probably act synergically against both pro and eukaryotic microbes. Synergic increase of activity: Xb=MPT-MRV – demanded by Worley et al. [17].

The growth inhibitory effect of various mixtures of carbendazim and furazolidone against Botrytis cinerea, Bipolaris zeicola (filamentous fungi) and Xanthomonas stewartii (bacterium) was shown in Figure 3. The synergic joint action was manifested in each case, and the ratio 1:1 was examined in primary screening by diffusion technique (Figure 2) proved to be synergic again. However, the optimum ranges were different for bacterium and fungi. Contrarily to fungi, where the synergy was more prominent with mixtures dominated by furazolidone, the carbendazim dominated mixtures were surprisingly more active against bacterium (compare ranges SI F and SI B on Figure 3). These ranges overlap between ratios [3C+2F] and [4C+1F] (C+F on Figure 3), and finally the composition containing [7C+3F] was chosen for development, and the effect of this mixture was studied in detail before preparing a marketable product.

Sensitivity of 53 phytopathogenic fungal species of various taxonomic positions was measured with agar diffusion technique, and 17 cases of significant synergy were manifested. However, the intensity of the improvement in activity varied within large limits (Figure 4). Outstanding synergy was revealed in the case of *Aspergillus versicolor* (orange rot), *Botrytis cinerea* (grape rot), *Colletotrichum dematium* (soya), Bipolaris sorokiniana (maize), Ustilago zeae (maize), Fusarium avenaceum (barley), *Fusarium oxysporum* (tomato). The diffusion technique results reliable data on qualitative character of joint action, but the variation coefficients in majority of cases are too high for getting the quantitative measures to support a claim in patent application, although, the qualitative data mark the level of prior art. Moreover, the activity of equal molar masses had been compared in diffusion tests, and the Patent Court required comparative data of equal masses of compounds in re Lemin's case [17]. Nevertheless, the experiment approved the broad spectrum antifungal activity of [7C+3F] mixture, and the results served for design of further experiments as well.

The antifungal activity was measured by agar diffusion technique: filter paper discs (5mm diameter) impregnated with 10.6M of test compound were placed centrally on the agar plate (one disc per dish of 90mm Ø) and diameter of growth inhibition zone was measured after 48 hours incubation at 22±1 °C. The territory of inhibited zone was

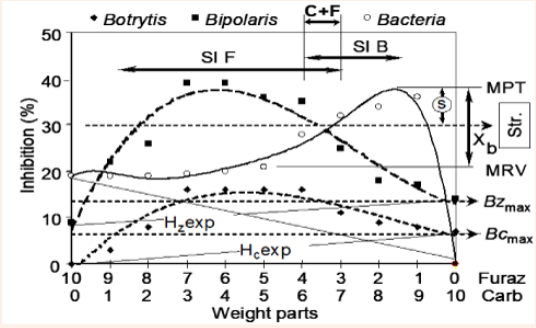

Figure 3: Joint action of furazolidone and carbendazim against selected phytopathogenic pro- and eukaryotic microbes.

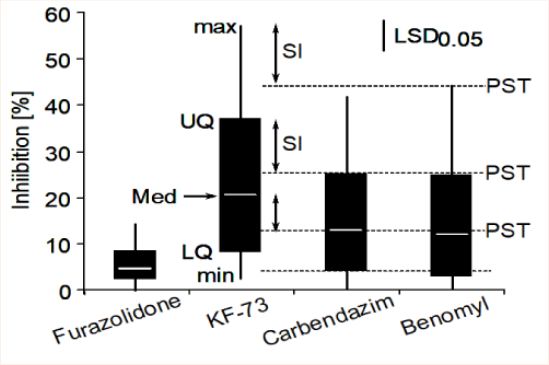

Figure 4: Antifungal spectrum of optimized binary mixture.

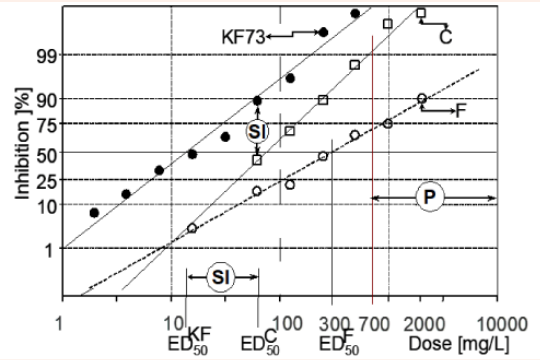

Figure 5: Joint action of furazolidone and carbendazim against *Blumeria graminis*.

calculated as a percent of the agar surface of Petri dish and taken as a rate of inhibition. Details of the method other than here were published earlier [16].

Abbreviations: KF-73 = {(0.134mg C + 0.068mg F) = 0.201mg = (0.3×10-6 M F + 0.7×10-6M C)} in the paper dics. In this model the effect of molar masses (equivalent number of molecules) of substances are compared. Box & Whishkers LQ and UQ = lower and upper quartiles, resp., Med=median, min and max = minimum and maximum value of synergy, resp.; PST = present state of technology as evaluated by median, upper quartile and maximum values (dotted lines), SI = range of significant synergic increase at $p<0.05$.

Most fungi examined in activity spectrum studies are either opportunistic or necrotrophic plant pathogens. The effect of [7C+3F] on biotrophic parasite was assayed in wheat/powdery mildew host/parasite system (Figure 5). The dose response lines (log/probit regression) fitted well to experimental values ($p<0.05$), and KF73 40wp was more effective as applied as leaf spray in each concentration level. However, the slopes of regression lines were different, thus the degree of synergy varied in concentration dependent manner. The mean values of the therapeutic index (T.I.) as evaluated by Sun's model [19] increased for example from 5.83 to 7.94 by decreasing the exposure of 60 mg/L to 6 mg/L of KF73. The increased activity can also be demonstrated by Colby's model [20] The synergic joint action expressed also in soil treatment (T.I.=2.9 at EC50), but did not appear in seed dressings (dose response lines are not shown).

The fully developed coleoptiles of wheat (Triticum aestivum L. cv Alcedo) seedlings infected with conidia of powdery mildew (Blumeria graminis) 24 hours earlier were sprayed run off with watery suspensions of fungicides by usual manner. The efficacy of treatments was evaluated counting colonies when the fungus started sporulate on control plants, and the inhibition was expressed by decrease of colony numbers as related to untreated control: The plants were maintained as described earlier [21].

Abbreviations: F, C and KF73 are dose response lines of treatments of carbendazim and furazolidone and their [7+3] mixture, each of them fits experimental data at $p<0.05$ level.

YC= 2.499X + 0.2796 (R^2=0.988) – EC50= 317.4 mg/L

YF= 1.4897x + 1.2733 (R^2=0.986) – EC50=77.5 mg/L

YC+F= 2.0347x + 2.6785 (R^2=0.982) – EC50=13.8 mg/L

Sun's T.I. = {(1/13.8)/([0.3/317.4]+[0.7/77.5])}=7.24

Colby's SI (%) = 15% > LSD0.05= 7% at 250 mg/L dose

Horsfall's SI (%)=9,8 % > LSD0.05= 7% at 250 mg/L dose

The arrow P marks the interval of phytotoxic effect of furazolidone. The horizontal and vertical arrows SI show the changes in either of the requested dose to attain 50 % of inhibition or the increase in efficacy caused by the same mass of preparates applied. The latter demonstrates that the synergy in this case stands requirements according to the decision of the US Patent Court in re Lemin's case [17].

The technical advance can be evaluated from various points of view, among them the most important is the effectiveness in improving the quality of agricultural products. The value of KF73 40 wp against seedborne pathogens was rated in the provocation field (Table 1), and anti-mildew effect was also examined. The combined preparate significantly proved to be more effective than the components alone, thus the synergy took place

Table 1: Curative effect (%) of microbicides in provocation field experiments.

Treatments			Pathogens			Vitality[c] %
No.	Compound	Rate of use (g a.i.)	leaves[a]	seeds[b]	seeds[b]	
			Erysiphe	*Fusarium*	*Xanthomonas*	
1	Control	0	0	0	0	24
2	Benomyl[d]	350	98	18	0	71
3	Streptomycin[e]	1000	0	0	44	59
4	Furazolidone[f]	400	35	10	31	46
5	Carbendazim[g]	350	82	15	0	55
6	3+2 (7:3)[h]	700	98	71	100	74
	LSD 5%		11	8	9	6

against powdery mildew in field conditions as well, but did not surpass benomyl, thus in this respect no technical advance was achieved. However, the ratio of seeds either infected with *Fusarium* or *Xanthomonas* in the crop dramatically decreased, the new preparate exhibited significantly higher efficacy, than either benomyl or streptomycin. This advance was reflected in the vitality of yield as well. The bacterial infection was absent; consequently, the high quality sowing seed was produced.

Seeds of *Triticum sativum L.* cv Alcedo were artificially infested with suspension of *Xanthomonas translucens* and dried up in room temperature, then subsequently dressed with appropriate doses of marketed pesticides (g a.i./t). Seeds were sowed in a provocation field and usual management for wheat was applied except fungicidal treatments. The experimental plots were sprayed with appropriate doses of the above preparates (g a.i./ha) at the start of flowering. The effectiveness of treatments was evaluated as follows:

a) The presence of powdery mildew infection was evaluated on coleoptiles. Hundred plants were examined counting the powdery mildew infected and free plants, and the percent ratio of mildew free seedlings was taken as protected against infection.

b) Ratio of uninfected seeds was determined after harvesting and the presence of pathogens in the spermosphere was examined by usual manners [22].

c) The harvested seeds were sowed in pots filled with sandy soil (100 per pot) and the seedlings with fully developed healthy coleoptiles were counted. Their percent ratio was taken as a vital one.

d) Treatments: Marketed products were applied according to the proposal of manufacturers (g a.i. per t or g a.i. per ha) to establish the present state of technology. d-Benlate 50 wp (DuPont,), e-Streptomycin pharmaceutical grade (Chinoin, Budapest, Hungary), f-Furoxon 40 wp (Chinoin, Budapest, Hungary), g- Kolfugo 25 fw (Chinoin, Budapest, Hungary), h- KF73 40 fw (special formula of PPI).

The usefulness of KF73 was also tested in a provocation experiment against seed borne bacterial blight disease as seed dressing (Table 2). The joint action was synergic and the rates stand requirements of Patent Office, thus results support a claim for patent application.

Seeds of *Oryza sativa L.* cv Lamont were artificially infested with suspension of *Xanthomonas oryzae pv. oryzae* and dried up at room temperature, and subsequently dressed with appropriate doses of uniformly formatted preparates. The plants were grown in pots filled with sandy soil (100 seeds per pot), and the effect of treatment was evaluated after full development of the first true leaf in control (untreated and uninfected seeds) pots. The visually indistinguishable of healthy control seedlings were counted, and their percent ration was taken as protected of infection. The rate of synergy (SI) was evaluated according to Horsfall's model [15]. Marketed preparates Furoxon 40 fw and Kolfugo 25 fw as standards for evaluation of technical advance. Furoxon 40 fw caused delayed germination, in this dose, but plant rapidly overgrow, and after formation of first true leaf were not distinguishable of untreated healthy ones. Comparing MIC values convincing synergic joint action was demonstrated (Table 3), the combination was 2-4 times more active than furazolidone or streptomycin and ten times more active than mercury chloride against *Erwinia* species. Seemingly, the optimum ratio established in Figure 3 is effective against a series of phytopathogenic species too. The poor activity of [C+F] against saprotrophic and symbiotic bacteria is a special advantage as compared to traditional microbicides used in controlling seed borne diseases. These data are supportive for Patent Application.

Table 2: Control of rice bacterial blight disease.

Treatments		Dose (g ai. per t seeds) + SI						
No.	Compounds[a]	300	SI	700	SI	1000	SI	LSD5%
1	Furazolidone	29		43		71[b]		9
2	Carbendazim	0		0		21		7
	1+2 (3:7)	43	+14	61	+18	96	+25	5

The antibacterial activity was determined by the traditional poisoned agar gel method using two fold dilution series of compounds, The appropriate amount of the compound was mixed with the agarized medium of proper composition before pouring into Petri dishes (l0 ml medium into a 90mm diameter dish). Then the agar plates were inoculated with bacterial suspensions using a multipoint inoculator. The colony growth was evaluated after 24h incubation at 20-22°C. The method was delineated in details in earlier publication [13]. None of the test bacteria was inhibited by 500 mg/L of carbendazim in the medium. The [C+F] mixture simultaneously inhibits pathogenic fungi and bacteria in the phytosphere, which property is its main advantage by means of comparative studies. There are more active fungicides in the market with antibacterial side effects (Figure 6), however, the noticeable synergy can not be demonstrated using them in mixtures. The azole fungicides, penconazole and prochloraz are highly active monosite inhibitors of large series of filamentous fungi without bactericidal side effect limited to some gram positive species, however the strength of this effect too weak to eliminate pathogens of spermosphere. Thus in this aspect [C+F] surpasses them, and this potency can be used to support a claim in patent application. Moreover, [C+F] breaked up the acquired tolerance of pathogens to generic agro-microbicides (Table 4).

Potential activities were calculated by Potency Mapping [23] based on activity of compounds measured at 10 mg/L concentration level in the medium against 42 fungal and 25 bacterial species, respectively, as described in legend of Figure 3. The height of grey columns is proportional to potential antifungal activity against filamentous fungi while the same of black columns against bacterial species: KF-73= carbendazim + furazolidone [7:3].

Arrows show differences in overall activities as related to the activity of combined substances (LSD0.05=1.7): A = KF-73 versus carbendazim, C = KF-73 versus furazolidone against fungi and B = KF-73 versus furazolidone against bacteria (carbendazim does not exhibited antibacterial activity). D = KF-73 versus streptomycin that means, the

Table 3: Joint action of furazolidone and carbendazim against bacteria.

Baktériumok	Minimum Inhibitory Concentrations (mg/L)			
	Strepomycin	KF73	Furazolidone	$HgCl_2$
Pathogen				
Agrobacterium tumefaciens	2-4	1-2	2-4	1-2
Erwinia amylovora	1-2	0.5-1	4-8	4-8
E. carotovora	2-4	0.13-0.25	1-2	1-2
Xanthomonas malvacearum	1-2	1-2	4-8	0.5-1
Corynebacterium michiganense	0.5-1	0.13-0.25	0.5-1	0.5-1
Saprobiont				
Erwinia herbicola	2-4	>100	>100	1-2
Pseudomonas fluorescens	4-8	>100	>100	2-4
Symbiont				
Rhizobium trifolii	0.5-1	>100	>100	2-4

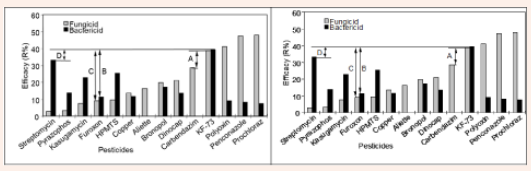

Figure 6: Antifungal spectrum of optimized binary mixture.

Table 4: Changes of sensitivity of phytopathogens due to acquired resistance to active substances. [a]= spontaneous mutant of ATCC 15580 strain; [b]=W – benomil sensitive; R - benomyl tolerant; [c]= KF 73 40 fw, 3+7 (w/w) mixture of furazolidone and carbendazim.

Host plant: *Malus domestica* cv Jonathan		Minimum Inhibitory Concentration (mg/L)		
Pathogen	Disease	KF73 40 fwc	Streptomycin	Benomyl
Erwinia amylovora	blossom blight	2	2	>100
E. amylovora - R[a]		2	>100	>100
Botrytis cinerea - W[b]	fruit decay	1	>100	0.5
B. cinerea - R	fruit decay	5	>100	>100
Venturia inaequalis -W	fruit scab	0.2	>100	1
V. inaequalis - R	leaf spor	1	>100	>100

antibacterial activity increased significantly as related to present state of technology, while the antifungal activity of KF-73 did not surpass top marketed fungicides, consequently, synergic increase realized only comparing to carbendazim, that means, this mixture can not be claimed as a fungicide in a patent application. Contrarily, the antibacterial effect of optimized mixture proved to be more prominent than that of streptomycin (the present state of technology), that means, this proof can support a claim to use it as an agro-bactericide.

The acquired tolerance often leads to complete loss of activity and it is the main factor of shortening the market life of synthetic pesticides. The optimized mixture [C+F] acted against various tolerant to agro-microbicides strains of both bacteria and fungi, which observation was a real breakthrough in process of development, as it was an unexpected phenomenon, thus the involvement of KF73 into pest management program is undisputable technological advance. The physiological basis of this action is most probably the metabolism of furazolidone in microbial cells that rapidly decompose this molecule. The main routes are: reduction of the nitro group or possible hydroxylation in ortho position to nitro group, and cleavage of the C=N bridge (Figure 7), then the opening of oxazolidine ring results a highly toxic metabolite, *β*-hydroxyethylhydrazine, which can inhibit various metabolic steps in the cell [24-26]. Data on mechanism of toxicity were obtained of clinical observations and experiments carried out with vertebrates (degradation scheme, binding to DNA, inhibition of monooxygenases and glucose-6-phosphate dehydrogenase), and only inhibition of phosphatidylcholine synthesis was demonstrated in fungal cells [25].

Analogies of medicine are frequently used in development of synthetic and botanical pesticides; however, the decision on therapeutic value of preparates is different. In medicine and veterinary, some iatrogenic effects might be accepted, for example, the drug applied more harmful to cancer cells than regular cells, or the use of arsenic derivatives to eliminate parasitic protozoans. In these cases usually, the ratio of ED50 or LD50 values is used to appreciate the therapeutic value. Contrarily, the adverse effects in the case of host/parasite pairs are rarely accepted, and the ratio of maximum tolerated dose by host plant and minimum inhibitory dose for pathogen should be taken (Table 5). Moreover, the

Table 5: The most important relationships to be evaluated for development and application of pest control agents. Therapeutic Index (T.I.) = MTDP/MIDC, where MTD and MID are maximum tolerated and minimum inhibitory doses of control agent, respectively.

Exposed organisms		Therapeutic index	Persistence (days)
To be controlled	To be protected (P)		
Traditional	*Homo sapiens*	no harm	not
	Host plant	>5	1-30
	Vertebrates	>100	not
	Bees	>100	not
Pest (C)	*Saccharomyces*	>3	not
Future	Symbionts	>10	not
	Antagonists	?	not
	Predators	?	not
	Ecosystems	?	?

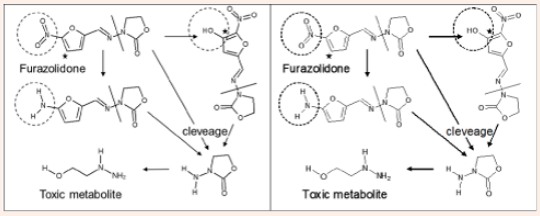

Figure 7: Deterioration of furazolidone.

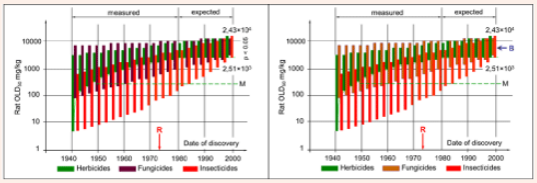

Figure 8: Changes in mammalian toxicity of pesticides.

decision maker should take account of suspected knowledge of users when recommends dose for practical applications, i.e., the three- to fivefold overdose cannot harm the exposed cultivated plant. The improvement of therapeutic value is an important issue, as it helps to convince Patent Examiners that the two most important criteria satisfied, namely, that the effect is novel and not obvious. Moreover, the mixture with improved therapeutic value as compared to its component obviously stands to criteria of technical advancement.

Both use and development of pesticides relates to environmental issues. as the synthetics have become the enemy number one by some social groups. The requirements in pest management practices inspired researches to improve toxicological properties of synthetic pesticides that resulted in astonishing changes in therapeutic values (Figure 8). The trends calculated from data obtained between 1940 and 1980 depicted the trend of development due to proper invariant principles of phytopharmaeutical sciences without intervention of outsiders (social activists, politicians, investors, etc.) that is a serious challenge to researchers and inventors working in this field of science. Nowadays, due to increased concerns about the harmful effect of residual, i.e., to consequences of chronic subtoxic exposures, it is highly advisable to change the used screening protocols and assess the mammalian toxicity in primary screening. Novel methods of the in silico drug design provide possibilities to size up the expected mammalian toxicity and to predict the fate of new molecules in biological conditions as well [27]. The risk of development can be reduced excluding candidates more toxic then 500 mg/kg. This limit was crossed first in development of herbicides in middle of past century (Figure 8), than followed a decade later by fungicides and finally by insecticides near 1980, indicating the difficulty to find proper target sites for selective inhibition that is related to similarities in physiology and cell biochemistry among various organisms. Unfortunately, the acquired tolerance to agrochemicals applied in pest management annuls easily all investments for development of new pesticide, so the probability of such mutations should also be tested in primary screening process; the requested level must be lower than 10-9 that is possible to measure in model experiments [28]. The trends were calculated from data obtained between 1940 and 1980, columns between the envelope second order polynomial functions mark the range between least and most toxic substances proper class ($p<0.05$).

Abbreviations: R – Emergence of acquired tolerance to carbendazim, M – the tolerated maximum toxicity for new discoveries, B – the toxicity of KF73 approached by means of Sun's model [19]. The object of our development, the [C+F] combination fits well to above criteria. The expected mammalian toxicity of KF73 can be approached following Sun's method:

$$LD_{\text{expected}} = [(a^C / LD50^C) + (b^F / LD50^F)] =][(0.7/10000) + (0.3/1508)] = 7450\ mg/kg\ rat$$

which means definitely lower toxicity than that of the furazolidone the more potent bactericide in KF73 40 fw, and can be accepted as a technical advancement. The therapeutic value of [C+F] can be demonstrated using data of Figure 5. The furazolidone per se proved to be phytotoxic to wheat leaves at 700 mg/L dose (=PTL), thus

$$T.I.^{KF} = (PTL^F / ED99^{KF}) = (700/197) = 3.54$$

that means, three times overdosing the KF73 will not harm the canopy of wheat. In general, this development can be considered as successful. The synergic character of joint action of carbendazim and furazolidone was demonstrated for a wide range of phytopathogenic bacteria and fungi, and the experimental proofs supported the claims of patent applications.

Methods proposed for demonstration of the character of joint action have been compared and evaluated:

a) The model of Colby [20] proved to be useless for comparison of the performance of compounds of highly different potency, thus it is proposed to be excluded of screening protocols,

b) The easy to handle model of *Horsfall and Dimmond* [15] suits to establish the optimum mixing ratios of binary mixtures, and data resulted of these experiments can be supportive for claims of patent application,

c) The Sun's model [19] is useful to compare dose response functions as well as to evaluate therapeutic value of examined substances,

d) The standardized agar diffusion technique is useful for qualitative detection of the character of joint action.

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Conflict of interest statement

The author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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