THE ANTIFUNGAL EFFECT OF SOME NITROPHENYL DERIVATIVES EXPRESSED IN WHEAT GERMINATION EXPERIMENTS

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In the germination experiments using 5 x 10^{-3} M aqueous solutions, several nitrophenyl derivatives such as p-nitroacetophenone, picric acid, 3,5-dinitrosalicylic acid, 2-oxo(4-nitrophenylacetic) acid, 2,4-dinitrobenzoic acid, 4-nitrobenzoic acid have been tested comparatively with 2-oxo-glutaryc acid, L-β-phenylalanine, 2,2'-bipyridine-3,3',6,6'-tetracarboxylic acid, and resorcinol. Most of 7-day old samples were fully contaminated with various fungi, except those treated with 2-oxo(4-nitrophenylacetic) acid, 4-nitrobenzoic acid, which seem to have an anti-fungal action. In addition, picric acid and 3,5-dinitrosalicylic acid inhibited much the germination process of wheat seeds, whereas 4-nitrobenzoic acid proved to have a stimulatory effect. Thus, the mean height of the lots treated with picric acid and 3,5-dinitrosalicylic acid solutions was 4.5 cm and 6.6 cm respectively, as compared to 4-nitrobenzoic acid treatment, which was 9.6 cm (more then the height of the blank-9.0 cm). Accordingly, the mass of the resulted plantlets was diminished by the picric acid treatment and stimulated by 4-nitro-benzoic acid (0.71 g/lot and 30.6 mg/plantlet in the case of picric acid; 2.15 g/lot and 60.8 mg/plantlet in the case of 4-nitro-benzoic acid).

***Key words**: Nitrophenyl derivatives; antifungal activity; wheat germination; flurescence; FTIR.*

Fungicides are used to fight fungal infections. They should only be applied when absolutely necessary, especially if they are in an at-risk group. Lowering the amount of fungicide in the environment lowers the selection pressure for resistance to develop [1-3]. In addition, the pathogens respond to the use of fungicides by evolving resistance. Therefore, new classes of chemicals should be tested for

antifungal activity. Dinitrophenols have also antifungal properties; however, Environment Protection Agency in SUA (EPA) included the dinitrophenols on the list of national priorities and in concentration of 3-46 mg dinitrophenol/kg body kill; no antidote is known (Dose max. admissible in water 70 ppb, EPA, 2004). It is assumed that dinitrophenols hinder the proton translocation through the mitochondrial inner membrane and therefore oxidative phosphorylation is inhibited (ATP is no longer formed and the cells deprive of essential energy supply). It is also possible that the dinitrophenols act toxically due to the inhibition of formation of some triplet states (instable biradicals) by a resonance process with the triplet structures in the living cells (A. Szent-Gyorgyi-Nobel Prize, 1937) [3-5]. Nevertheless, some other compounds might have antifungal activity, especially those containing nitrogen atom or amino groups. We also observed that some nitrophenyl derivatives possess an antifungal activity. Hence, we tried in this paper to test their antifungal action and to bring information related to their biological mechanism of activity.

The biological activity of some synthetic compounds containing the di- and nitrophenyl moiety has been compared in wheat germination experiments with that of some well-known metabolic inhibitors and stimulators. In addition, fluorescence quenching and infrared spectra of the investigated compounds were brought to clarify the biological events.

MATERIALS AND METHODS

Chemical reagents. The reagents were of analytical purity (Merck, Sigma, Chimopar) and the solution and the water slurries were prepared using redistilled water.

Solutions and suspension for treatment. Several aqueous solutions of nitroderivatives were prepared, having concentrations of $5x10^{-3}$ M. The following compounds were tested: 1. picric acid; 2. p-nitroacetophenone; 3. 2,2'-bipyridine-3,3',6,6'-tetracarboxylic acid (BP-TCA); 4. 3,5-dinitrosalicylic acid; 5. 2-oxo(4-nitrophenylacetic) acid; 6. 2,4-dinitrobenzoic acid; 7. 2-oxo-glutaryc acid; 8. 4-nitrobenzoic acid; 9. L-phenylalanine; 10. resorcinol. A water-based blank was also introduced.

Biological material. The wheat samples (Triticum aestivum), Henika variety, were taken from the Agricultural Research Station of Suceava. The 1000 seeds weighed 37.2 g and had a residual humidity of 12%.

Equipment. The chemical syntheses were carried out using the organic chemistry lab equipment of the Chemistry Department of "Al. I. Cuza" University of Iasi. The experiments and the germination determinations were performed in Petri dishes, on double Watmann no. 1 filter paper at room temperature. The separation and purification of the compounds obtained were carried out using thin layer chromatography on silica gel (Kieselgel $60F_{254}$, Merck) and on silica gel column. The infrared spectra were obtained using a Jasco FT/IR660 Plus Fourier spectrometer in the range from 0 to 15000 cm^{-1}.

Fluorescence measurements Steady state fluorescence measurements were conducted with a Kontron SFM25 spectrofluorimeter equipped with thermostated cell holders and temperature was kept constant by a circulating water bath. The protein concentration ranged from 0.2 to 10 μM and most of measurements were performed with 1 μM. The excitation wavelength was set at 380 nm and the data were collected at

520 nm (typically for an oxidized flavin). The experiments were performed at 25 °C by using a 1 cm sealed cell (total volume 3 ml) and corrected for background signal.

Procedure. The germination was determined according to ISTA recommendations (Seed Science and Technology, 1993), but we also used 50-seed lots, which germinated on filter paper, in Petri dishes, in three repetitions. The first count took place after three days (energy of germination, EG), the second after 7 days (germination rate, GR). The treatment lasted for an hour, then the seeds were laid as uniformly as possible in Petri dishes, on double filter paper, together with the treatment solution. The seeds with a visible root were considered germinated. The seeds were watered daily with 5 ml of bidistilled water. After 7 days, the plantlets were cut at the level of the seeds, measured and weighed (height, H, in cm and mass, m, in grams).

Statistics. The results were processed using the Tukey test [6].

RESULTS AND DISCUSIONS

Most samples were fully contaminated with various fungi, except those treated with 2-oxo(4-nitrophenylacetic) acid, 4-nitrobenzoic acid, which seem to have an anti-fungal action (Figure 1). We supposed that nitro groups are reduced to the amino ones, which manifest an antifungal effect. In this case, 4-nitrobenzoic acid could be reduced to p-aminobenzoic acid, which is a vitamin with real stimulatory activity for the wheat plantlets. The most active substance in these experiments proved to be phenylalanine, which increased slightly by 8% the total height of the plantlets as compared to the blank and by 13% their mean weight. Picric acid and 3,5-dinitrosalicylic acid inhibited much the germination process of wheat seeds, whereas 4-nitrobenzoic acid proved to have a stimulatory action (*table 1)*.

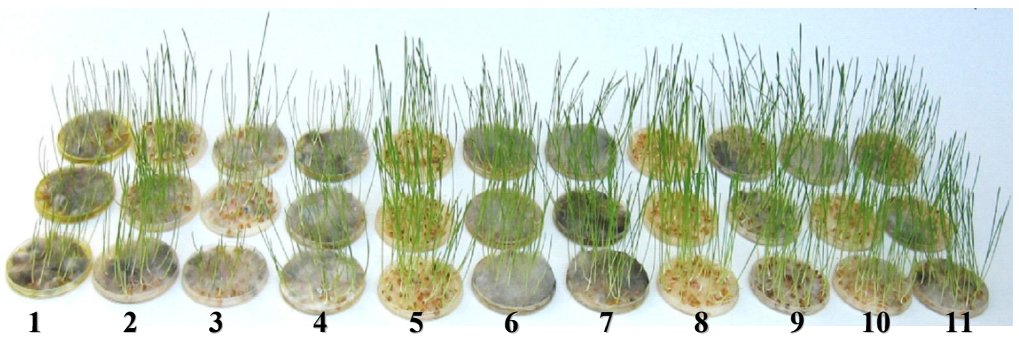

Figure 1 **The biological effect of some nitrophenyl derivatives and other compounds on wheat germination. Some of them proved to have an antifungal activity, whereas the others a toxic or even a stimulatory one. 1 – picric acid; 2 – p-nitroacetophenone; 3 – 2,2'-bipyridine-3,3',6,6'-tetra-carboxylic acid; 4 – 3,5-dinitrosalicylic acid; 5 – 2-oxo (4-nitrophenylacetic); acid; 6 – 2,4-dinitrobenzoic acid; 7 - 2-oxo-glutaryc acid; 8 – 4-nitrobenzoic acid; 9 – L-phenylalanine; 10 – Resorcinol; 11 – Blank (water).**

Thus, the mean height of the lots treated with picric acid and 3,5-dinitrosalicylic acid solutions was 4.5 cm and 6.6 cm respectively, as compared to 4-nitrobenzoic acid treatment, which was 9.6 cm (more then the height of the

blank-9.0 cm). Accordingly, the mass of the resulted plantlets was diminished by the picric acid treatment and stimulated by 4-nitro-benzoic acid (0.71 g/lot and 30.6 mg/plantlet in the case of picric acid; 2.15 g/lot and 60.8 mg/plantlet in the case of 4-nitro-benzoic acid).

Table 1

The biological effect of some nitroderivatives on wheat germination

Treatment	Germi-nation Rate (G.R.)	Total height of plantlets of the lot (**H**, cm)	Plantlets size (**S**, cm)	Plantlets mass (**M**, g)	Average mass of plantlets (**m**, mg)
1 – picric acid	72%	143.2±50.9	4.5±1.1	0.71±0.1	30.6±2.4
2 – p-nitroacetophenone	87%	262.4±21.5	6.9±0.3	1.53±0.1	40.6±2.7
3 – BP-TCA	94%	134.7±32.2	3.5±1.9	0.97±0.1	24.3±2.5
4 – 3,5-dinitrosalicylic acid	77%	223.4±13.1	6.6±2.3	1.26±0.1	37.3±4.3
5 – 2-oxo(4-nitrophenylacetic) acid	80%	330.6±14.9	9.1±0.1	2.06±0.3	56.7±1.9
6 – 2,4-dinitrobenzoic acid	92%	323.7±39.1	7.8±0.4	1.86±0.2	44,9±3.3
7 – 2-oxo-glutaryc acid	85%	279.9±10.3	8.0±0.5	1.47±0.1	42.3±4.0
8 – 4-nitrobenzoic acid	73%	340.2±14.9	9.6±0.2	2.15±0.1	60.8±4.6
9 – L-β-phenylalanine	91%	388.0±8.1	9.7±0.2	2.16±0.1	53.9±2.0
10 – Resorcinol	96%	416.6±34.5	9.4±0.6	2.35±0.2	52.9±2.1
11 – Blank, H_2O	99%	393.0±53.5	9.0±0.2	2.08±0.3	47.4±2.2
D (Tukey test)	9.5	67.5	0.8	0.4	9.3

*) Average of three independent values.

Fluorescence quenching of E376H-MCAD enzyme, which contain riboflavin as prostetic group (1,1 μM) was tested using nitroderivatives in 100 mM phosphate buffer (pH 7.0; 2 % glycerol; 25 ºC). MCAD is an acyl-CoA dehydrogenase acting on medium chain length Acyl-CoA. 2,4-Dinitro-*ortho*-cresol (DNOC), 2,5-dinitrophenol (2,5-DNF) p-nitrobenzoic acid (pNBA), as well as p-nitrobenzoyl acyl CoA, ligand of MCAD (pNBzCoA) were investigated (Figure 2). The intense quenching activity of DNOC, more powerful than that of 2,5-dinitrophenol was associated with a stronger uncoupling ability. 4-Nitrobenzoic acid did not manifest a similar effect, although it contains an ionisable group.

FT-IR spectra (*figure 3*) of the investigated compounds showed characteristic and intense absorption patterns at wave numbers higher than 6000 cm^{-1}, which correspond to an amount of energy as high as that of ATP formation.

Nevertheless, uncouplers had a higher absorption over 6000 cm^{-1} as compared to phenylalanine or 2,4-dinitrobenzoic acid.

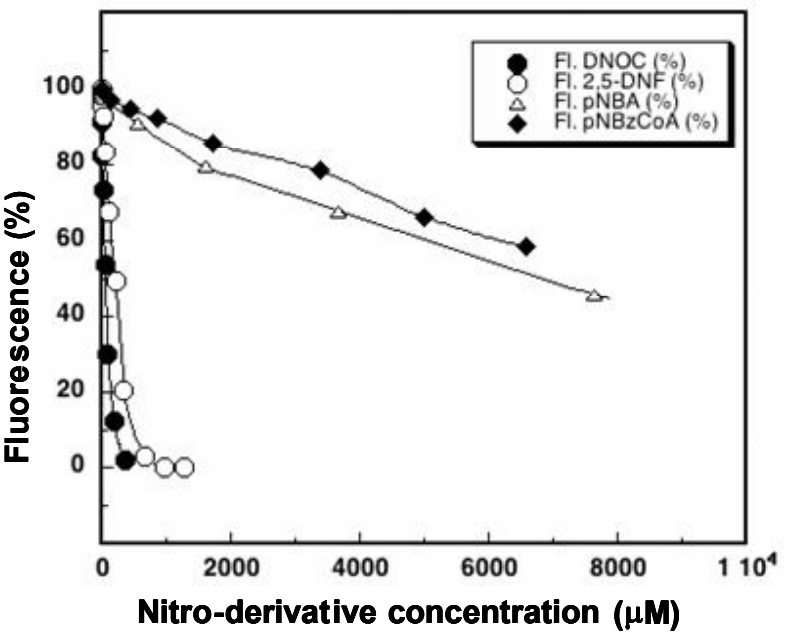

Figure 2 **The effect some nitroderivatives on the fluorescence of Acyl -CoA dehydrogenase . Both 2,4 -dinitro-*ortho*-cresol and 2,5 -dinitrophenol inhibited strongly the fluorescence, whereas 4 -nitro-benzoic acid as well as p-nitrobenzoyl -CoA showed a moderate inhibition.**

CONCLUSIONS

Some nitrophenyl compounds proved to have antifungal effect in simple experiments of germination. Possibly, the reduction of nitro groups to amino ones could be related to the antifungal effect. Dinitrophenyl derivatives with uncoupling activity may also have a large absorption band in infrared region aver 6000 cm^{-1} as well as fluorescence quenching of Acyl-CoA dehydrogenase. Therefore, the mechanism biological activity as well as that of toxicity might be related to energy transfer in ATP formation and not with proton translocation through the biological membranes. Further research is still necessary to clarify all aspects of the biological activity of di- and nitrophenols.

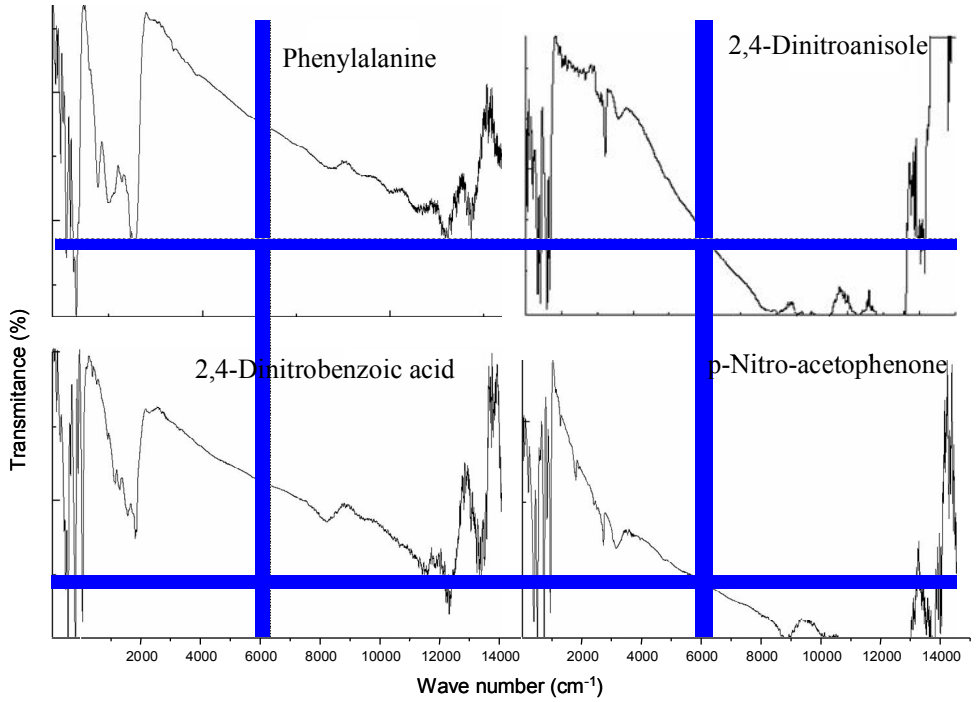

Figure 3 **FT-IR spectra of the investigated uncouplers show characteristic and nse absorption patterns at wave numbers higher than 6000 cm^{-1}, which correspond to an amount of energy as high as that of ATP formation. Contrary, phenylalanine and 2,4-dinitrobenzoic acid, which are not uncouplers have less intense absorbance at 6000 cm^{-1}.**

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