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## Fungitoxic Evaluation of New Modified Amidophosphonates (AP1, AP2) on the *in vitro* Growth of Two Fungal Strains

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### ABSTRACT

We performed this work to highlight the *in vitro* antifungal properties of two amidophosphonates newly synthesized (AP1, AP2). These molecules were synthesized from amino esters and chloroacetyl chloride in two steps using the Michaelis-Arbuzov reaction. We have selected after testing several concentrations 15, 20 and 25  $\mu\text{M}$  for AP1, 10, 25, 40  $\mu\text{M}$  for AP2. The study of the antifungal power by solid medium-diffusion method was performed after microscopic study and purification of fungal isolates from wheat leaves hard, give us the opportunity to identify two fungi: *Septoria tritici* and *Aalternaria tenuis*. Results show an antifungal power of two molecules, the growth inhibition-percentage is higher among *Aalternaria tenuis*. In addition, AP2 molecule appears to have a stronger antifungal activity. Determining the Minimum Inhibitory Concentration (MIC) by the dilution method in liquid medium, shapeless on the effectiveness of our molecules which is to order of 40  $\mu\text{M}$  (AP1) and 25  $\mu\text{M}$  (AP2) for *Septoria tritici*, 25  $\mu\text{M}$  (AP1) and 15  $\mu\text{M}$  (AP2) for *Alternaria tenuis*.

**Key words:** *Septoria tritici*, *Aalternaria tenuis*, amidophosphonate, MIC, Antifungal activity

### INTRODUCTION

Molecules with phosphonate group are widely studied in biochemistry and medicine (Groutas *et al.*, 1998). They saw applications inbound as antiviral agents (Snoeck *et al.*, 2002), antibiotics (Johnstone and Rose, 1979), neuroatcif (Ortalo-Magne *et al.*, 2005), herbicides (Matsuno-Yagi and Hatefi, 1993), fungicides (Maier and Diel, 1994) and cancer (McGuigan *et al.*, 2005), (Fig. 1). Chemical control is the most means effective short-term. Since, 1930s, the evaluation of thousands of new molecules has led to the gradual development of active ingredients more efficient (Serghat *et al.*, 2004). Phosphonates are distinguished by their excellent efficiency regarding diseases caused by various Phycomycetes as an example *Phytophthora* and their biological properties, as a chemical agents "Alternative", acting not on the pathogen it self but involving the defense mechanisms of the host plant (Guest *et al.*, 1988). If their activities in the fight against lower fungi (Oomycetes) are recognized, they could also be used against other classes of fungi (Barchietto, 1989; Bompeix, 1989). The evaluation of the potential biological activity, in particular pesticide of amidophosphonates is causing by combination phosphonyl-carboxamide (Kafarski *et al.*, 1985). Given the importance of these compounds, we were interested in this study to two new molecules (AP1 and AP2) containing amidophosphonate moiety, synthesized by application of the Arbuzov reaction using triethylphosphite (Guezane *et al.*, 2012; Arbuzov, 1906).

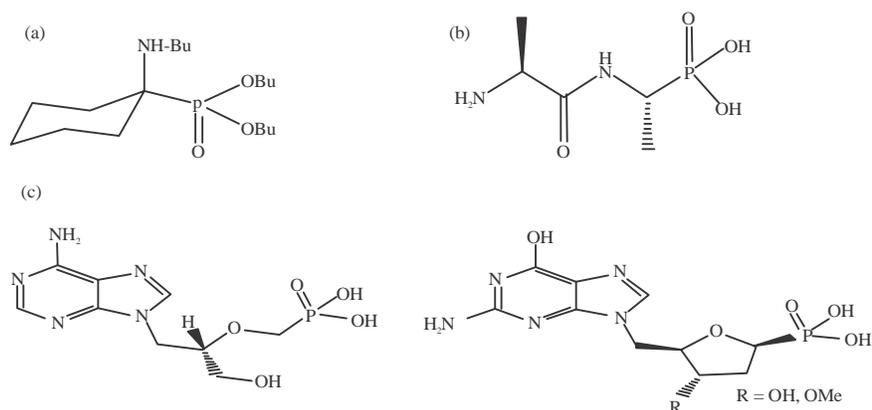


Fig. 1(a-c): Chemical structure of some phosphonates (a) Herbicide, (b) Antibiotics and (c) Antiviral

In this context, the main aim of this study is to evaluate the antifungal potential of these synthetic amidophosphonates and evaluate their effects on the growth of two fungal isolates which cause hard wheat leaf diseases: *Septoria tritici* and that of *Aalternaria tenuis*. Wheat is a cereal that has an important place in Algeria and is the backbone of the food system (Khalil, 1977) and its infestation of mold can cause significant losses by reducing its quality and/or quantity (Terrain and Graallet, 2003) and search alternatives to better protect seeds should be taken into consideration.

## MATERIALS AND METHODS

**Synthesis of  $\alpha$ -amidophosphonates (AP1, AP2) by the michaelis-arbuzov reaction:** The starting chiral methyl-chloroacetamide alkyl esters were easily prepared in excellent yield (90-95%) by treatment of the chiral amino esters with chloroacetyl chloride in the presence of Triethylamine (TEA) in Tetrahydrofuran (THF) at 0°C, followed by the Michaelis-Arbuzov reaction with triethyl phosphite gave the chiral methyl 2-(diethoxyphosphoryl)acetamide 2-alkylacetate AP1 et AP2 in (88-90%) yield (Fig. 2).

**Preparation of AP1 and AP2 solutions:** We prepared solutions of 10 mL from 0,232 g to the AP1 and 0,114 g of the AP2. We also dissolved these molecules in acetone (2%) and after several tests in the laboratory, we have selected concentrations of 10, 25 and 40  $\mu$ M for AP1 molecule and 15, 25 and 40  $\mu$ M for AP2 molecule.

**Biological material:** Biological material used in this study is represented by the strain of *Septoria tritici* and *Alternaria tenuis* isolated from wheat leaves of a crop field between November, 2012 and April, 2013, identified and analyzed at the mycology laboratory of the National Institute of Plant Protection (NIPP). This identification is based on macroscopic criteria (general appearance of colonies) and microscopic (Study of vegetative filaments, fruiting bodies and spores etc.) (Chabasse, 2002).

**Culture medium:** The strain of *Septoria tritici* and *Alternaria tenuis*, were revived in petri dishes of 90mm of diameter on Malt agar medium at 20 mL per dish, incubated in climatic chamber at 20 and 22°C to darkness for 7 days (Champion, 1997).

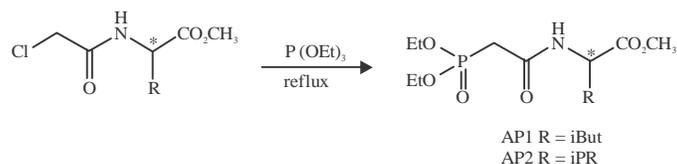


Fig. 2: Synthesis of  $\alpha$ -amidophosphonates

**Evaluation of antifungal activity of AP1 and AP2 molecules:** Antifungal activity was evaluated by solid medium-diffusion method (method of disks). Measuring diameter of inhibition pushed mushrooms, used to calculate the MIC (minimum inhibitory concentration) and to evaluate the percentage of inhibition relative to the activity of a reference antifungal marketed in Algeria: Moncozeb (Delahousse, 2003).

**Determination of diameters of inhibition zones:** We used solid medium-diffusion method as reported by Rasooli *et al.* (2008) Paper discs whatman No. 40 at 6 mm of diameter previously prepared and impregnated with different concentrations of AP1 (15, 20 and 25  $\mu$ M) and AP2 (10, 25 and 40  $\mu$ M), are placed on the agar previously seeded to suspension of *Septoria tritici* and *Alternaria tenuis*, from a pre-culture of 2 days. The absence of mycelial growth is reflected by a translucent halo around the disc, whose diameter is measured using a graduated rule (including the disc diameter 6 mm). We also used Moncozeb as fungitoxic reference (positive control), distilled water for negative control and control acetone at 2%. Incubation is realized in to climatic chamber at temperature of  $20 \pm 2^\circ\text{C}$  for 7 days. Experiment was performed in triplicate (03 replicates for each).

**Determining the rate of inhibition:** The growth filaments is daily recorded and measurement of diameters of the two colonies was performed at the end of incubation for calculating the rate of inhibition (I%) (Laib, 2012).

$$I(\%) = \frac{dC - dE}{dC} \times 100$$

I(%) = Inhibition rate expressed as percentage

dC = Diameter of colonies for control petri dishes

dE = Diameter of colonies for tested petri dishes

**MIC determination by the method of broth dilution:** We have used concentrations presenting percentages of inhibition higher or equal to 50%. The 100  $\mu$ L of our solutions with different concentrations were added to 900  $\mu$ L of middle liquid malt-agar containing the test strain. The tubes thus prepared are incubated at  $20 \pm 2^\circ\text{C}$  for 7 days. After incubation, the tubes in which no overall growth of mold are identified: MIC is the minimum concentration for which no overall mold growth compared to the control without product, it provides information on the effectiveness of molecules studied (Rotimi *et al.*, 1988).

**Statistical analysis:** Statistical analysis was performed by Student's t-test using the software MINITAB Eng Version 13.31 (Dagnelie, 1999).

## RESULTS

**Determination of the diameters zones of inhibition:** Table 1 shows the evaluation of the antifungal activity of AP1 and AP2 molecules. We note that for the strain of *Septoria tritici*, diameter of inhibition is 15 mm from 10  $\mu$ M of AP1, whereas for strain of *Alternaria tenuis*, diameter of inhibition is 12.4 mm from 10  $\mu$ M and reach a maximum of 22.33 mm at 40  $\mu$ M, in contrast to the positive control, inhibitor effect is much more marked. However negative control and control acetone have no inhibitory effect (0 mm). For AP2 molecule, we see an inhibitory effect of the reference molecule (positive control) ranging to 22.5 mm to *Septoria tritici* until 23.3 mm for *Alternaria tenuis*. By against, inhibitory effect of AP2 seems stronger for the strain of *Alternaria tenuis* with inhibition diameters varying between 14 and 23.98 mm for different concentrations used. Negative control and acetone control show no inhibitory effect (0 mm).

**Determining the rate of inhibition:** From Table 2 We note that the negative control (distilled water) and the acetone control show no inhibition rate on the strain of *Septoria tritici* and *Alternaria tenuis* (null Activities). by against the AP1 and AP2 molecules had varying activities. Concerning *Alternaria tenuis*, AP1 molecule gave greater than 50% inhibition rate at concentrations of 25 and 40  $\mu$ M, these rates are highly significant ( $p \leq 0.01$ ) relative to that of the positive control. However, *Septoria tritici* has proved less sensitive with one very significant inhibition rate ( $p \leq 0.01$ ) of 50.12% for concentration of 40  $\mu$ M. *Alternaria tenuis* proved most sensitive to AP2 molecule, despite using lower concentrations than that used for the AP1 molecule, very highly significant inhibition rate ( $p \leq 0.01$ ) higher than 50% were found for all three concentrations used. Parallel for strain of *Septoria tritici*, very highly significant inhibition rate ( $p \leq 0.01$ ) greater than 50% are marked only for concentrations of 20 and 25  $\mu$ M.

**Determination of MIC:** Table 3 shows the Minimum Inhibitory Concentrations (MIC) of molecules AP1 and AP2 on both fungal strains studied. We're interested with inhibitions rate over

Table 1: Average diameters of inhibition zones (mm $\pm$ SD) of *Septoria tritici* and *Alternaria tenuis* treated with AP1 and AP2 molecules

Parameters	Zones of inhibition (mm) <i>Septoria tritici</i>	Zones of inhibition (mm) <i>Alternaria tenuis</i>
<b>AP1</b>		
Positive control	22.20 $\pm$ 0.65	23.00 $\pm$ 1.02
Negative control	0	0
Acetone control	0	0
10 $\mu$ M	15.00 $\pm$ 0.3	12.40 $\pm$ 0.3
25 $\mu$ M	16.07 $\pm$ 0.24	21.83 $\pm$ 0.7
40 $\mu$ M	17.57 $\pm$ 0.24	22.33 $\pm$ 0.22
<b>AP2</b>		
Positive control	22.50 $\pm$ 0.6	23.30 $\pm$ 0.2 mm
Negative control	0	0
Acetone control	0	0
15 $\mu$ M	17.20 $\pm$ 0.7	14.00 $\pm$ 0.3
20 $\mu$ M	21.73 $\pm$ 1.3	22.55 $\pm$ 0.3
25 $\mu$ M	22.08 $\pm$ 0.6	23.98 $\pm$ 0.3

Table 2: Inhibition rate I' (%) of AP1 and AP2 towards the two strains tested

I' AP1 (%)	Negative control	Acetone control	Positive control	10 $\mu$ M	25 $\mu$ M	40 $\mu$ M
	<i>Septoria tritici</i>	-	0	58.54 $\pm$ 0.24	45.45 $\pm$ 0.22	47.40 $\pm$ 0.24
<i>Alternaria tenuis</i>	-	0	73.33 $\pm$ 0.03	49.77 $\pm$ 0.12	70.73 $\pm$ 0.06	71.84 $\pm$ 0.07
I' AP2 (%)	Negative control	Acetone control	Positive control	15 $\mu$ M	20 $\mu$ M	25 $\mu$ M
	<i>Septoria tritici</i>	-	0	59.09 $\pm$ 0.56	49.45 $\pm$ 0.63	57.69 $\pm$ 0.16
<i>Alternaria tenuis</i>	-	0	74.00 $\pm$ 0.10	51.11 $\pm$ 0.05	54.55 $\pm$ 0.03	59.95 $\pm$ 0.30

Table 3: MIC of molecules (AP1, AP2) in liquid medium

MIC ( $\mu\text{M}$ )	<i>Septoria tritici</i>	<i>Alternaria tenuis</i>
AP1	40	25
AP2	25	15

50%. The MIC obtained varied based molecules and fungal species. The AP1 is active molecule on the growth of two fungal strains with respective MIC at 40 and 25  $\mu\text{M}$ . However, AP2 molecule has greater activity with MIC of 25  $\mu\text{M}$  for strain of *Septoria tritici* and 15  $\mu\text{M}$  for strain of *Alternaria tenuis*.

## DISCUSSION

Synthetic antifungal agents are applied at large scale for prevent mycelial growth (Prasad *et al.*, 2010). The difficulty of developing an antifungal molecule is linked on the one hand to the ultra structure of the fungal cell that has three barriers: the chitinous cell walls, membrane ergosterol and eukaryotic nucleus (Chami, 2005). On the other hand, antifungal molecules themselves that can cause resistance (Prasad *et al.*, 2010; Prasad and Kapoor, 2004). The disk diffusion method allowed us to demonstrate antifungal power of our two  $\alpha$ -amidophosphonates modified (AP1 and AP2) regarding the tested fungal strains. One of factors influencing intensity of antifungal action is the dose, it was generally observed *in vitro* on antifungal activity of the essential oil (Do Amaral *et al.*, 1998; Evans and Martin, 2000; Castillejos *et al.*, 2006). Results obtained in this study shows a decrease in mycelial growth of *Alternaria tenuis* and *Septoria tritici*, this reduction is dependent on dose of AP1 and AP2 molecules, with zones of inhibition more marked by the treatment with Moncozeb. This is due to the fact that some fungicides are acting on the energetic system of fungal cells by inhibiting the respiratory process, on the other are acting on the synthesis of the constituents of the fungus. Substances have yet aim to disrupt the cells and their divisions within fungal fabric (Rocher, 2004). This inhibition may also be due to the prevention of conidial germination: an essential first step in the sequence of operations leading up to the establishment of germinative tube and the hyphae, thereafter. The process begins by hydration followed by lytic enzymes action such as chitinase and  $\alpha$  and  $\beta$ -glucanases. This lead to decomposition of the cell wall of conidia thickened to allow the appearance of the initial digestive tract. Once this event occurs, there is a balance between the lytic systems and synthetic of enzymes necessary for the normal extension of the hyphae. An imbalance in one or the other enzymes system leads to the inhibition and/or prevention of growth (McEwan, 1994). In the case of amidophosphonates (oranophosphorus), the molecules used may have an inhibitor power of lipid peroxidation identical to that resulting from the activity of tolclofos-methyl cited in the works of Rocher (2004) used against the *Rhizoctonia* of potato, further phosphonates are at the origin of a phosphate deficiency in fungi causing the synthesis of substances inducing elicitation in the host plant (Leroux, 2003a, b). The phosphonate is a strongly systemic product: he travels the whole plant by the two streams of the sap (Leconte *et al.*, 1988). It indirectly stimulates the defense system of plants. Indeed, in Arabidopsis, has been shown that fosetyl induces expression of PR-protein PR-1 involved in the systemic acquired resistance (Chuang *et al.*, 2000). It has been demonstrated by the work of Bompeix *et al.* (1981) and Saindrenan (1990) that the phosphonate is the active metabolite of fosetyl-Al, systemic fungicide. His behavior is different from that of fungicides at direct action: it discreetly disrupts the metabolism of pathogens agents and leads to hyper-production of secondary metabolites elicitors of plant defense reactions. Our results point in the same sense that those of Zahri *et al.* (2008) who have highlighted the manifestation of a good antifungal potency against the wheat *Septoria* due to *Septoria tritici*, under effect of other

fungicides including triazoles, carbendazimes and carbamate derivatives. Strain of *Alternaria tenuis* appears to be most sensitive to amidophosphonates tested, this result is comparable to the work of Bouguerra (2012), whose the genus *Alternaria* is the most sensitive to the essential oil of fennel seeds with a total inhibition  $4000 \mu\text{g mL}^{-1}$ . Laib (2012) have shown that the genus *Alternaria* isolated from chickpeas has a sensitivity towards essential oil of dry flowers of *Lavandula officinalis*. Mancozeb or monoalkyldithiocarbamate usually used against Helminthosporium diseases, the rusts and rots, also shows a significant fungicidal action on *Septoria tritici* and *Alternaria tenuis*. Mancozeb contact fungicide has a preventive activity, its fungitoxic effect was attributed mainly to its ability to generate isothianate which inactivates the thiol group of enzymes and metabolites in cells (Ragsdale, 1994). The rates of inhibition and determination of MIC of Amidophosphonates on fungal strains by the method of broth dilution, confirm the results obtained in solid medium. According to the results, rates of inhibition greater than 50% are characterized by the activity of the molecule AP2 with the best MIC ranging to  $25 \mu\text{M}$  for strain of *Septoria tritici* and  $15 \mu\text{M}$  for strain of *Alternaria tenuis*. The difference in sensitivity may be due to some factors, namely the type of target microorganism (Magan and Olsen, 2004) and the difference between the molecular structure of the AP1 and AP2 molecule and their mode of action.

## CONCLUSION

Results obtained in this work shows fungitoxic effect of the two molecules newly synthesized (AP1, AP2) to mold hard wheat. When the highlight of this activity, it was found that these molecules have varying activities against testing strains and *Alternaria tenuis* is the most sensitive strain. In addition, AP2 seems to be the most active molecule. This should be taken into consideration in the design of future fungicides.

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