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Some Plant Defense Stimulators can induce IL-1β production in human immune cells *in vitro*

Lény Teyssier[a], Gabriele Sorci[b], Johanna Chluba[c], Sébastien Aimé[d], David Wendehenne[d], Olivier Lamotte[d], Jean-Louis Connat[a,*]

[a] Université de Bourgogne-Franche-Comté, UMR INSERM U866 Lipide Nutrition Cancer, F-21000, Dijon, France
[b] Biogéosciences, CNRS UMR 6282, Université de Bourgogne-Franche-Comté, 6 boulevard Gabriel, F-21000, Dijon, France
[c] INSERM U1231, Univ. Bourgogne, Université de Bourgogne-Franche-Comté, F-21000, Dijon, France
[d] Agroécologie, AgroSup Dijon, CNRS, INRA, Univ. Bourgogne, Université de Bourgogne-Franche-Comté, F-21000, Dijon, France

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ABSTRACT

Among Plant Protection Products (PPP), a new emerging category of pesticides act by stimulating plant defense in order to improve plant resistance against microbial pathogens. Given that these compounds, the so-called Plant Defense Stimulators (PDS) act on innate immunity, we tested, using an *in vitro* approach on human mononuclear leucocytes (PBMC), the potential toxicity (XTT assay) and inflammatory effects (production of IL-1β) of 4 PPP belonging to different chemical families. We found that two products (LBG-01F34® and Regalis®) did not induce any cytotoxicity or IL-1 β production. The product BION-50 WG®, that contains Acibenzolar-S-methyl (ASM) and silica particles did not present any cytotoxicity but induced a significant increase in the production of the inflammatory cytokine IL-1 β. Finally, Vacciplant® that contains laminarin, was highly cytotoxic and pro-inflammatory. It induced a strong production of IL-1 β when used at a concentration in the culture medium, as low as 0.02 mg/mL. We also tested the potential toxic effect of these 4 PPP on 4 days old zebra fish larvae. After 24 h of exposure, our results indicate that Vacciplant® induced zebra fish larvae mortality at concentration of 20 µg/mL. LBG did not induced significant mortality at concentrations up to 1 mg/mL whereas Regalis was lethal for 0,3 mg/mL concentrations and BION-50 WG began to induce mortality at 2,5 mg/mL.

Our results indicate possible effects of PDS on IL-1β production in human cells and fish survival, calling for more studies on the potential noxious side effects of these compounds.

1. Introduction

Plant Defense Stimulators (PDS) are developed by agrochemistry as an alternative to classical pesticides that are highly criticized for their potential toxicity and impact on the environment. Various PDS are, thus, available for applications to numerous crops.

The mode of action of PDS has been suggested to be specific to the plant physiology. For instance, acibenzolar-S-methyl (ASM), the active molecule of BION 50 WG®, belongs to the benzothiadiazole family and is an analogue of the plant hormone salicylic acid (SA). ASM stimulates or primes the plant immune response in several plant species such as wheat (*Triticum aestivum*) ([1–3] and reference therein). A similar effect has been reported for phosphite, the active substance of LBG-01F34®. When used in pre-treatment, its primes the defense response induced by SA, inducing a stronger resistance of plants to pathogens. In potato, phosphite treatment restricts the development of the oomycete *Phytophthora infestans* likely through a SA-dependent process [4]. In Vacciplant®, the active principle is laminarin, an oligosaccharide extracted from *Laminaria digitata* alguae. Laminarin reduces the soft rot disease symptoms induced by the bacterial pathogen *Erwinia carotovora* subsp. *carotovora* in tobacco [5]. In grape, it was shown to induce defense responses including the production of reactive oxygen species, defense gene expression, phytoalexin production and to reduce infection by *Botrytis cinerea* and *Plasmopara viticola*, the causal agents of grey mold and mildew, respectively [6]. Finally, Prohexadione, the active compound of Regalis®, is a molecule that inhibits giberellic acid biosynthesis, a typical plant hormone, and thus delays growth of apple tree. However, prohexadione has also a PDS activity. Treatment of apple trees with prohexadione induces activation of defense gene and a reduction of apple scab [7]. Furthermore, Regalis® reduces the occurrence

Abbreviations: PDS, Plant Defense Stimulator; PPP, Plant Protection product; PBMC, peripheral blood mononuclear cells; ASM, acyl-benzolar-S-methyl

* Corresponding author.
E-mail address: jean-louis.connat@u-bourgogne.fr (J.-L. Connat).

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of fire blight caused by *Erwinia amylovora* and induces plant defense [8]. Given these modes of actions, it is generally thought that PDS might be safer than the classical pesticides used until now.

We recently showed that the PDS Stifenia® (or FEN560) can potentially induce an inflammatory response in humans [9]. Indeed, this product stimulates the production of inflammatory cytokines in human leucocytes *in vitro* and upregulates the expression of TNF-α and IL-1β in zebra fish larvae. In the present paper, we investigated the effects of four other commercially available PPP on the production of the pro-inflammatory cytokine IL-1β in human peripheral blood mononuclear cells (PBMC). In addition to the direct effect of PPP on IL-1β production, we also investigated a possible additive or synergistic effect between PPP and the bacterial wall component lipopolysaccharide (LPS) which is known to induce a strong pro-inflammatory response [9,10]. Finally, we tested the different compounds on 4 days old larvae of the fish *Danio rerio* to get information about the toxic doses on small aquatic animals.

2. Material & methods

2.1. Chemicals

BION-50 WG®, LBG-01F34®, Regalis® and Vacciplant® were purchased from Dijon Céréales (France), a provider for farmers. These compound are only partially soluble in water. Compounds were prepared at 17 mg of active substance /mL in RPMI medium (human cell cultures) or water (zebra fish tests), and gently shaken for 30 min. After centrifugation to remove insoluble particles (30 min at 20,000 *g*), no pellet was detected only for LBG-01F34®. Supernatants of these different stock solutions were diluted in the assay medium to reach the tested concentrations that were chosen according to recommended use (Table 1). LPS from *Escherichia coli* 0111:B4 stock solution (1 $mg.mL^{-1}$ in pure water), purchased from Sigma-Aldrich, was diluted in RPMI to reach a final concentration of 10 $ng.mL^{-1}$ in cell culture.

2.2. Human peripheral blood mononuclear cells

Buffy coats from healthy donors were obtained from Etablissement Français du Sang (EFS, Besançon, France, Agreement N°DECO-14-0124). Peripheral blood mononuclear cells (PBMC) were prepared using Pancoll (density 1.077 $g.mL^{-1}$, PAN-biotech Gmbh, Germany) and Blood Sep Filter tubes (Dominique Dutscher, France). Briefly, 15 mL of Pancoll were collected into the lower part of a Blood Sep Filter tube by a short centrifugation. Then, 25 mL of buffy coat and 15 mL of DPBS (Dulbecco's Phosphate-Buffered Saline, PAN-biotech Gmbh, Germany) were added, gently mixed and centrifuged (400 *g*, 30 min, room temperature) without brake for the deceleration phase. The PBMC ring was collected, washed 3 times in DPBS without Ca^{2+} and Mg^{2+} and centrifuged (300 *g*, 10 min, 4 °C). Cells were suspended in 2 − 5 mL of DPBS depending on the size of the cell pellet and kept on ice. Viable PBMC were counted using trypan blue, suspended in RPMI medium supplemented with 10 % BSA Bovine Serum Albumin, w/v and 1% PSA Penicillin 10000 $units.mL^{-1}$, Streptomycin 10 $mg.mL^{1}$, Amphotericin B 25 $\mu g.mL^{-1}$ prepared in water) and then seeded at the density of 10^5 cells per well in 150 µL of medium in 96-well plates.

2.3. Treatments

Because PBMC are floating cells, cells were seeded in 96 wells round bottom plates (Nunc®, Corning Inc.) (10^5 cells per well) then treated by various concentrations of the different compounds dissolved in 10 µl vehicle (RPMI) within one hour after plating. The final well volume was adjusted to 170 µl and cells incubated for 20 h.

For each dose tested, 8 replicates from at least 3 different healthy human blood donors were used. These 8 replicates were tested for cell metabolic activity (XTT) and IL-1β production, as described below.

2.4. Cell metabolic activity

Cell metabolic activity was determined using the XTT (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay (Sigma-Aldrich, France) as already described in Teyssier et al. [9]. After centrifugation of the plates and removing of supernatant for IL-1β measurement, cells pellets were suspended in the reaction mixture (100 µL RPMI without phenol red and 20 µL containing 0.9 mg/mL XTT and 0.01 mM PMS) and incubated at 37 °C for 4 h. Absorbance was measured at 490 nm with a background subtraction at 660 nm using a microplate reader (Infinites M200 PRO, Tecan, France). The results are expressed as percentage of metabolic activity compared to control non-exposed cells.

2.5. Quantification of IL-1β

Production of the inflammatory cytokine IL-1β was measured on aliquots of the culture medium from each well 20 h after the treatment using ELISA assay (Human IL-1β ELISA Ready-SET-Go! eBiosciences, France) according to the supplier's instructions. Reference curves were run in each sample plates. Each reference curve was established with

Table 1
Technical data concerning formulated products tested in this study.

Formulated product	Active substance and concentration in formulated product	EFSA journal	Recommended use in field treatment	Concentration of active substance[a]	AOEL[b]	Fish toxicity[c]
BION 50GW	Acibenzolar-S-methyl, 500 g/kg	2014;12(8):3691	3.2 − 5 g/L (0.32 % - 0.5 %, m/v [d])	1.6 - 2.5 g/L	0,03 mg/kg bw/day	1.1 mg/L
LBG-01F34	Potassium phosphonates, 730 g/L	2012;10(12):2963	67.2 − 336 mL/L (6,72 % - 33,6 %, v/v [e])	49.05–245.3 g/L	5 mg/kg bw/day	> 118 mg/L
Regalis	Prohexadione, 100 g/kg	2010; 8(3):1555	0.3–12.5 g/L (0.03 % - 1.25 %, m/v [f])	0.03 - 1.25 g/L	0,35 mg/kg bw/day	> 100 mg/L
Vacciplant	Laminarine, 37 g/L	2017;15(6):4836	1 - 3 mL/L (0.1 % - 0.3 % [g])	0.037 - 0.111 g/L	NR[h]	> 88 µg/L

[a] Concentration of active compound regarding recommended concentration use.
[b] AOEL: acceptable operator exposure level (mg/kg of body weight/day) for active substance as mentioned in EFSA article.
[c] Fish toxicity for active substance on *Oncorhynchus mykiss* (rainbow trout): acute toxicity (LC_{50}) at 96 h as mentioned in EFSA article.
[d] ANSES n°2011-0627.
[e] ANSES n° 2002-0556.
[f] ANSES n°2012-1952.
[g] ANSES n°2012-2220.
[h] not required.

Table 2
Linear mixed effect models exploring the effect of four plant protection products (LBG-01F34®, Regalis®, BION-50WG®, Vacciplant®) on cell metabolic activity (expressed as percent change with respect to the control group). Each model included the experimental group and the replicate nested within the experimental group as fixed effects and the blood donor identity as a random effect. The model investigating the effect of LBG-01F34® included 6 experimental groups (control, LBG-01F34® 0.1 mg/mL, LBG-01F34® 0.1 mg/mL + LPS, LBG-01F34® 0.3 mg/mL, LBG-01F34® 0.3 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of Regalis® included 6 experimental groups (control, Regalis® 0.1 mg/mL, Regalis® 0.1 mg/mL + LPS, Regalis® 0.3 mg/mL, Regalis® 0.3 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of BION-50 WG® included 8 experimental groups (control, BION-50 WG® 0.1 mg/mL, BION-50 WG® 0.1 mg/mL + LPS, BION-50 WG® 0.3 mg/mL, BION-50 WG® 0.3 mg/mL + LPS, BION-50 WG® 1 mg/mL, BION-50 WG® 1 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of Vacciplant® included 6 experimental groups (control, Vacciplant® 0.02 mg/mL, Vacciplant® 0.02 mg/mL + LPS, Vacciplant® 0.06 mg/mL, Vacciplant® 0.06 mg/mL + LPS, LPS) and 8 replicates per experimental group. Degrees of freedom were approximated using the Sattherwaite method.

	Plant protection product											
Metabolic activity (XTT)	LBG-01F34®			Regalis®			BION-50WG®			Vacciplant®		
Fixed effects	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*	*df*	*F*	*p*
Experimental group	7,126	9.38	< 0.001	5,94	5.56	< 0.001	7,189	30.62	< 0.001	5,94	93.72	< 0.001
Replicate within Experimental group	56,126	0.99	0.513	42,94	0.53	0.988	56,189	0.88	0.700	42,94	0.15	1.00

	Plant protection product											
Metabolic activity (XTT)	LBG-01F34®			Regalis®			BION-50WG®			Vacciplant®		
Random effect	*estimate ± SE*	*z*	*p*	*estimate ± SE*	*z*	*p*	*estimate ± SE*	*z*	*p*	*estimate ± SE*	*z*	*p*
Blood donor	512.97 ± 519.21	0.99	0.162	474.07 ± 482.57	0.98	0.163	343.76 ± 283.60	1.21	0.113	156.58 ± 162.94	0.96	0.168
Residual	399.05 ± 50.28	7.94	< 0.001	407.88 ± 59.49	6.86	< 0.001	228.61 ± 23.52	9.72	< 0.001	305.20 ± 44.52	6.86	< 0.001

standard human IL-1β solution (500 pg/mL) extemporaneously prepared and sequentially diluted twice seven times in the assay buffer to reach 3.9 pg/mL. Duplicates of each concentration were run and Optical Density (OD) of the samples were plotted on a double logarithmic scale. Extrapolation of the concentrations of IL-1β in the measured cells supernatants was done in the linear part of the reference curve using an algorithm. For high concentrations (more than 400 pg/mL) samples were diluted to fit with the medium part of the reference curve.

2.6. Zebrafish strains, maintenance and treatment

According to the European Union Directive 2010/63/EU, no specific ethics approval was required for this project, as all zebra fish larvae used in this study were less than 120-h post-fertilization (hpf) old. Wild type fishes (AB strain) were obtained from the ZIRC (Oregon, USA) and kept at 28 °C with a light:dark cycle 14:10 h. They were fed twice a day with dried flake food (Gemma Mirco, Skretting, France). The fish were mated and spawning was stimulated by the onset of light. Zebrafish eggs were collected immediately after being fertilized and distributed in 6-wells plates (20 eggs/well containing 5 mL Eggwater). At 4 days post-fertilization, water was replaced by fresh water containing the desired concentration of tested compound and incubated during 20 h. At least four replicates of 20 zebrafish larvae per condition were conducted.

2.7. Statistical analyses

We used general linear mixed effect models (PROC MIXED, SAS) to investigate the effect of the four PPP on PBMC production of IL-1β, and on cell metabolic activity.

IL-1β had a highly right-skewed distribution and was log-transformed to normalize the distribution of model errors. In each model, the dependent variable was the log-transformed IL-1β, while experimental group and replicate nested in the experimental group were included as fixed terms. We also included cell metabolic activity as a covariate in each model to correct for a possible covariation between metabolic activity and IL-1β production. Finally, the identity of blood donor was included as a random factor to take into account the repeated nature of the data per donor. Degrees of freedom were approximated using the Sattherwaite method. Pairwise post-hoc comparisons were conducted on between group differences of least squares means and p values adjusted for multiple comparisons using the Scheffé method.

Cell metabolic activity was expressed as percent change with respect to the control group, and analyzed with linear mixed effect models that included experimental group and replicate nested in the experimental group as fixed factors, and blood donor identity as a random factor. Degrees of freedom were approximated using the Sattherwaite method. Pairwise post-hoc comparisons were conducted on between group differences of least squares means and p values adjusted for multiple comparisons using the Scheffé method.

The effect of PPP on the mortality of zebra fish larvae was tested using a non-parametric test (Kruskal-Wallis test). For each product concentration, we computed the proportion of dead larvae per replicate, and these values were used as the dependent variable in the model.

3. Results

The formulated products we tested are either sold as powders (BION-50WG®, Regalis®) or as liquid preparations (LBG-01F34®, and Vacciplant®) ready to be dissolved in water. However, most of them are not totally soluble in water. To study their effects on human PBMC or zebra fish larvae, we used aqueous soluble extracts obtained as described in Section “Materials and Methods”. According to French Agency for Food, Environmental and Occupational Health and Safety (ANSES) documents (Table 1), we tested concentrations in the range of the recommended use-concentrations of these formulated products (Table 1, column 5).

3.1. Effect of LBG-01F34®

3.1.1. Cell metabolic activity

The linear mixed effect model indicated that PBMC in the different experimental groups had significantly different metabolic activity (Table 2). However, pairwise post-hoc comparisons showed that PBMC exposed at the three LBG doses (0.1, 0.3, and 1 mg/mL) had similar metabolic activity compared to the control group (Fig. 1A; table S1). While PBMC treated with LPS had similar metabolic activity compared

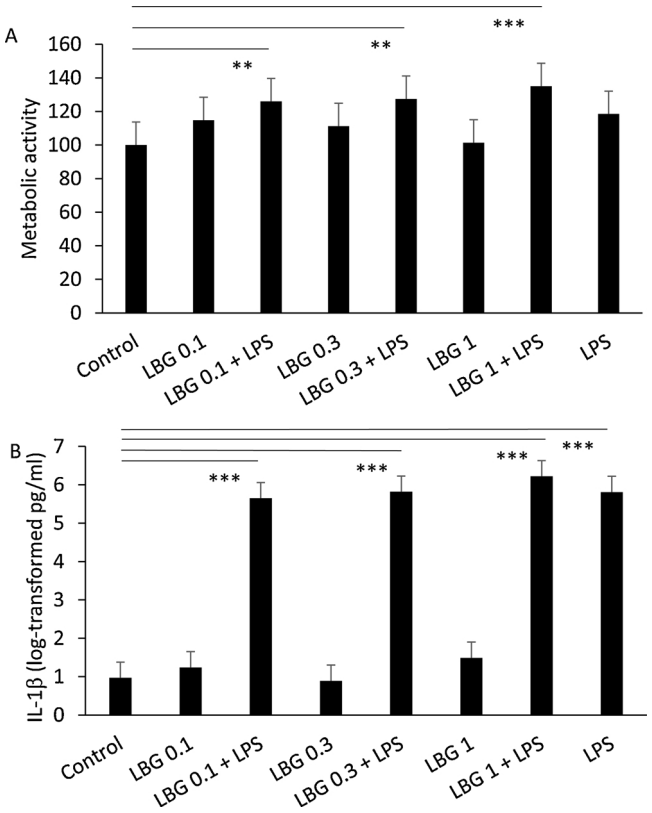

Fig. 1. Least squares means (+ SE) of metabolic activity (A) and IL-1β production (B) of human PBMC exposed to three doses of LBG-01F34® (0.1, 0.3, 1 mg/mL) alone or with LPS. Least squares means of metabolic activity were computed from a linear mixed effect model where the experimental group and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Least squares means of IL-1β production were computed from a linear mixed effect model where the experimental group, metabolic activity and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Metabolic activity is expressed as percent changes with respect to the control group, and IL-1β as log-transformed pg/mL.
Only statistically significant differences between the control and the treatment groups are indicated. The full details of all pairwise comparisons are reported in the online table S1 and S2.
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

to the control group, PBMC treated with both LPS and LBG (at the three doses) had statistically higher metabolic activity compared to the control group (Fig. 1A; table S1).

There was no variation among replicates within experimental groups nor among blood donors in metabolic activity (Table 2).

3.1.2. IL-1β

The linear mixed effect model indicated that PBMC in the different experimental groups produced significantly different amount of IL-1β, and that, across groups, metabolic activity was positively correlated with IL-1β production (Table 3). Pairwise post-hoc comparisons showed that PBMC exposed at the three LBG doses had similar production of IL-1β compared to the control group (Fig. 1B; table S2). PBMC treated with LPS produced more IL-1β compared to the control group (Fig. 1B; table S2); however, adding LBG (at the three doses) to LPS-treated PBMC did not alter the IL-1β production compared to PBMC that were only exposed to LPS (Fig. 1B; table S2).

There was no variation among replicates within experimental groups nor among blood donors in IL-1β production (Table 3).

3.2. Effect of Regalis®

3.2.1. Cell metabolic activity

The linear mixed effect model indicated that PBMC in the different experimental groups had different metabolic activity (Table 2, Fig. 2A). However, pairwise post-hoc comparisons showed that PBMC exposed to the two Regalis® doses (0.1 and 0.3 mg/mL) had similar metabolic activity compared to the control group (Fig. 2A; table S3); the only statistically significant pairwise comparison was between metabolic activity of PBMC treated with Regalis® at the dose of 0.3 mg/mL and PBMC treated with LPS (Fig. 2A; table S3).

There was no variation among replicates within experimental groups nor among blood donors in metabolic activity (Table 2).

3.2.2. IL-1β

The linear mixed effect model indicated that PBMC in the different experimental groups produced significantly different amount of IL-1β, and that, across groups, metabolic activity was positively correlated with IL-1β production (Table 3). Pairwise post-hoc comparisons showed that PBMC exposed to the two Regalis® doses had similar production of IL-1β compared to the control group (Fig. 2B; table S4). PBMC treated with LPS produced more IL-1β compared to the control group (Fig. 2B; table S4); however, adding Regalis® (at the two doses) to LPS-treated PBMC did not alter the IL-1β production compared to PBMC that were only exposed to LPS (Fig. 2B; table S4). There was no variation among replicates within experimental groups nor among blood donors in IL-1β production (Table 3).

3.3. Effect of BION-50WG®

3.3.1. Cell metabolic activity

The linear mixed effect model indicated that PBMC in the different experimental groups had different metabolic activity (Table 2, Fig. 3A). Pairwise comparisons showed that PBMC treated with BION-50 WG at the 0.1 and 0.3 mg/mL had similar metabolic activity compared to the control group (Fig. 3A; table S5); while PBMC treated at the highest BION-50 WG dose (1 mg/mL) had slightly lower metabolic activity compared to the control group (Fig. 3A; table S5). PBMC treated with LPS had higher metabolic activity compared to the control group (Fig. 3A; table S5). PBMC treated with both LPS and BION-50 WG had lower metabolic activity compared to LPS-only treated PBMC (Fig. 3A; table S5 except at the dose of 0.3 mg/mL where PBMC treated with LPS and BION-50 WG had a reduced metabolic activity (Fig. 3A; table S5).

There was no variation among replicates within experimental groups nor among blood donors in metabolic activity (Table 2).

3.3.2. IL-1β

The linear mixed effect model indicated that PBMC in the different experimental groups produced significantly different amount of IL-1β, and that, across groups, metabolic activity was negatively correlated with IL-1β production (Table 3). Pairwise post-hoc comparisons showed that, whatever the dose, BION-50 WG induced an up-regulation of IL-1β production compared to the control group (Fig. 3B; table S6). LPS induced a strong up-regulation of IL-1β production (Fig. 3B; table S6). PBMC treated with both LPS and BION had much higher IL-1β production compared to the control group (Fig. 3B; table S6); whereas the pairwise comparisons between IL-1β production of PBMC co-treated with BION-50 WG and LPS *vs.* LPS alone showed that only at the dose of 0.3 μg/mL the difference was statistically significant (co-treated PBMC producing lower amount of IL-1β compared to LPS-only) (Fig. 3B; table S6).

There was no variation among replicates within experimental groups nor among blood donors in IL-1β production (Table 3).

Table 3
Linear mixed effect models exploring the effect of four plant protection products (LBG-01F34®, Regalis®, BION-50WG®, Vacciplant®) on PBMC IL-1β production (log-transformed). Each model included the experimental group, the metabolic activity, and the replicate nested within the experimental group as fixed effects and the blood donor identity as a random effect. The model investigating the effect of LBG-01F34® included 6 experimental groups (control, LBG-01F34® 0.1 mg/mL, LBG-01F34® 0.1 mg/mL + LPS, LBG-01F34® 0.3 mg/mL, LBG-01F34® 0.3 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of Regalis® included 6 experimental groups (control, Regalis® 0.1 mg/mL, Regalis® 0.1 mg/mL + LPS, Regalis® 0.3 mg/mL, Regalis® 0.3 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of BION-50 WG® included 8 experimental groups (control, BION-50WG® 0.1 mg/mL, BION-50WG® 0.1 mg/mL + LPS, BION-50WG® 0.3 mg/mL, BION-50WG® 0.3 mg/mL + LPS, BION-50 WG® 1 mg/mL, BION-50 WG® 1 mg/mL + LPS, LPS) and 8 replicates per experimental group. The model investigating the effect of Vacciplant® included 4 experimental groups (control, Vacciplant® 0.02 mg/mL, Vacciplant® 0.02 mg/mL + LPS, LPS) and 8 replicates per experimental group. Degrees of freedom were approximated using the Sattherwaite method.

	Plant protection product															
IL-1β	LBG-01F34®				Regalis®				BION-50WG®				Vacciplant®			
Fixed effects	*estimate ± SE*	*df*	*F*	*p*	*estimate ± SE*	*df*	*F*	*p*	*estimate ± SE*	*df*	*F*	*p*	*estimate ± SE*	*df*	*F*	*p*
Experimental group		7,125	124.56	< 0.001		5,93.2	48.09	< 0.001		7,189	149.27	< 0.001		3,63	382.38	< 0.001
Metabolic activity	0.016 ± 0.004	1,119	14.46	< 0.001	0.023 ± 0.006	1,91.4	13.05	< 0.001	−0.043 ± 0.006	1,176	47.97	< 0.001	0.022 ± 0.003	1,63	56.53	< 0.001
Replicate within Experimental group		56,125	0.38	1.00		42,93	0.27	1.00		56,188	0.48	0.999		28, 63	1.08	0.386

	Plant protection product											
IL-1β	LBG-01F34®			Regalis®			BION-50WG®			Vacciplant®		
Random effect	*estimate ± SE*	*z*	*p*	*estimate ± SE*	*z*	*p*	*estimate ± SE*	*z*	*p*	*estimate ± SE*	*z*	*p*
Blood donor	0.38 ± 0.41	0.93	0.176	0.79 ± 0.87	0.92	0.180	0.76 ± 0.64	1.18	0.120	0		
Residual	0.94 ± 0.12	7.90	< 0.001	1.66 ± 0.24	6.82	< 0.001	1.77 ± 0.18	9.70	< 0.001	0.38 ± 0.07	5.61	< 0.001

3.4. Effect of Vacciplant®

3.4.1. Cell metabolic activity

A preliminary experiment was performed using treatments of PBMC cultures with concentrations of 0.03, 0.1, 0.3 and 1 mg/mL Vacciplant for 20 h (Suppl. Fig. 1). Results indicated that this compound was highly cytotoxic for the two most concentrated solutions, inducing a decrease of 80 % of the XTT metabolism compared to controls. We thus investigated lower ranges of concentrations, namely 0.02, 0.06 and 0.2 mg/mL on four different cells batches. The 0,2 mg/mL concentration induced again high cytotoxicity (data not shown) and we thus excluded this data to only keep the 2 other concentrations (Fig. 4A). The linear mixed effect model indicated that PBMC in the different experimental groups (0.02 and 0.06 mg/mL) had different metabolic activity (Table 2, Fig. 4A). Pairwise post-hoc comparisons showed that, at both doses, Vacciplant had a negative effect on cell metabolic activity, compared to the control group (Fig. 4A; table S7); the difference in metabolic activity was particularly strong when PBMC were exposed to the 0.06 mg/mL dose (-76 %), suggesting a severe toxic effect on cell viability at this dose. PBMC treated with LPS had similar metabolic activity compared to the control group (Fig. 4A; table S7); while PBMC treated with both LPS and Vacciplant had lower metabolic activity compared to both control and LPS-treated PBMC (Fig. 4A; table S7).

Cell metabolic activity did not vary among replicates within experimental groups nor among blood donors (Table 2).

3.4.2. IL-1β

Given the strong negative effect of Vacciplant on cell viability at the highest dose (0.06 mg/mL), its effect on IL-1β production was investigated at the 0.02 mg/mL dose only.

The linear mixed effect model indicated that PBMC in the different experimental groups produced significantly different amount of IL-1β, and that, across groups, metabolic activity was positively correlated with IL-1β production (Table 3). The pairwise comparison showed that PBMC treated with Vacciplant produced significantly more IL-1β compared to the control group (Fig. 4B; table S8). PBMC treated with LPS had a much higher production of IL-1β compared to the control group (Fig. 4B; table S8). PBMC treated with both LPS and Vacciplant produced more IL-1β when compared to the control group and similar amount of IL-1β when compared to PBMC treated with LPS alone (Fig. 4B; table S8).

There was no variation among replicates within experimental groups, while the estimated covariance parameter for blood donors was null (Table 3).

3.5. Toxicity test on zebrafish larvae

LBG-01F34 did not induce any mortality on zebra fish larvae (only at the highest concentration tested, 2.5 % of the larvae died but this was not significantly different from the control, non-exposed group) (Table 4). BION-50 WG had a moderate effect on zebra fish mortality with 6% of larvae dying at the highest concentration tested (Table 4). On the contrary both Regalis and Vacciplant had a strong effect on zebra fish mortality, with 100 % of the larvae dying at the concentration of 0.3 mg/ml and 0.02 mg/ml, respectively (Table 4).

4. Discussion

Plant Protection Products (PPP) that we studied in this report act as plant defense stimulator and thus stimulate the plant innate immunity. The goal of this new agrochemical approach is to limit classical pesticide extensive use, as to reduce the noxiousness for human beings and the environment. However, innate immunity shares common pathways between plants and animals [11,12] and the compounds that are developed to stimulate plant defenses might also have side effects on animals and humans. In agreement with this view, we recently reported one of such side effects of the PDS Stifenia® on human PBMC and zebra fish larvae [9]. Stifenia® induced an over-production of the pro-inflammatory cytokine IL-1β, decreased the viability of cultured human PBMC, and activated the expression of inflammatory genes in zebra fish larvae. Here, we tested four additional PDS for which the biologically active compounds are known.

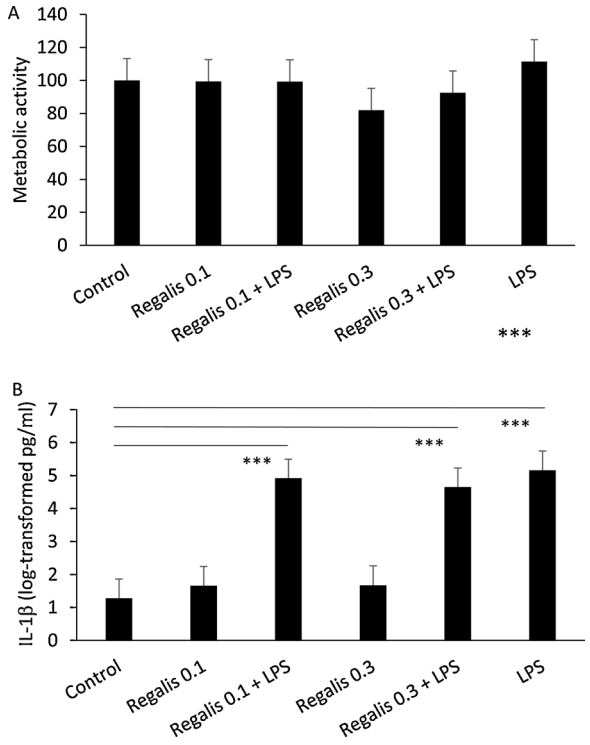

Fig. 2. Least squares means (+ SE) of metabolic activity (A) and IL-1β production (B) of human PBMC exposed to two doses of Regalis® (0.1, 0.3 mg/mL) alone or with LPS. Least squares means of metabolic activity were computed from a linear mixed effect model where the experimental group and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Least squares means of IL-1β production were computed from a linear mixed effect model where the experimental group, metabolic activity and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Metabolic activity is expressed as percent changes with respect to the control group, and IL-1β as log-transformed pg/mL.
Only statistically significant differences between the control and the treatment groups are indicated. The full details of all pairwise comparisons are reported in the online table S3 and S4.
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

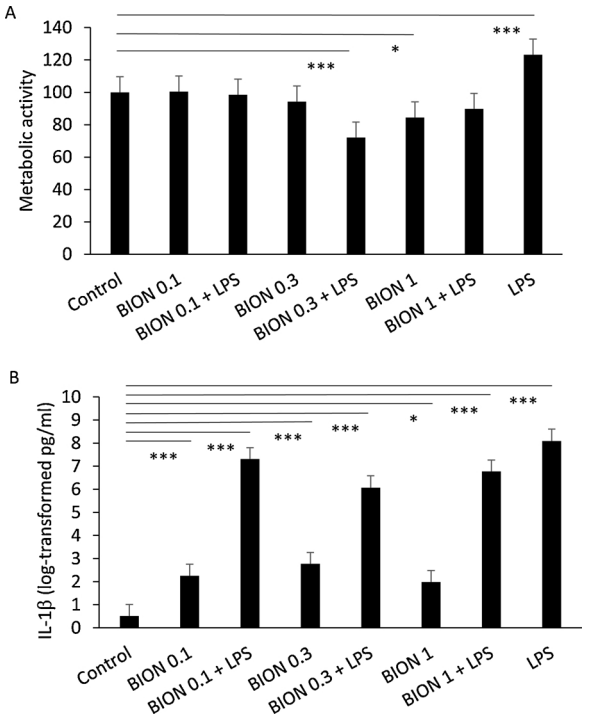

Fig. 3. Least squares means (+ SE) of metabolic activity (A) and IL-1β production (B) of human PBMC exposed to three doses of BION-50 WG® (0.1, 0.3, 1 mg/mL) alone or with LPS. Least squares means of metabolic activity were computed from a linear mixed effect model where the experimental group and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Least squares means of IL-1β production were computed from a linear mixed effect model where the experimental group, metabolic activity and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Metabolic activity is expressed as percent changes with respect to the control group, and IL-1β as log-transformed pg/mL.
Only statistically significant differences between the control and the treatment groups are indicated. The full details of all pairwise comparisons are reported in the online table S5 and S6.
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

Our data investigated metabolism of XTT by human PBMC during 4 h after PPP treatments. This measurement of cellular metabolic activity could be considered as an indicator of cell viability. We also measured the released IL-1β in the culture medium, an indicator of the activation of the NLRP3 inflammasome involved in the innate immunity of humans [11]. NLRP3 is expressed in leucocytes [13,14] and is activated upon stimulation by various stimuli involved in innate immunity [15,16]. Upon activation, NLRP3 protein complex binds caspases-1 that cleave IL-1β inactive precursor to its short active form released outside of the cells. IL-1β is thus only produced by activated leucocytes. Since the antibody used in ELISA could cross-react with the non cleaved precursor form of IL-1β stored in the cytosol, it cannot be excluded that leaking of this form from cells presenting plasma membrane deterioration by the PPP could slightly surestimate the measured cytokine concentrations, especially when metabolic activity is found to be low. However, measure of IL-1β in the medium clearly indicates a cellular dysfunction.

Among the four PDS tested here, two of them, LBG-01F34® and Regalis®, did not have any effect on PBMC metabolic activity and IL-1β production *in vitro*. They neither synergize the inflammatory effect of LPS on the cells. Viability of PBMC treated by 1 mg/mL LBG-01F34® estimated with Trypan blue was not different from control (data not shown) and confirm the absence of toxicity at this concentration. These compounds might however have other effects on eukaryotic cells. On one hand, phosphite (phosphonate) contained in LBG-01F34® is an ionic structure that naturally combines with sodium and sulfates. Metabolomic analysis of phosphite-treated *Arabidopsis thaliana*plants showed changes in several metabolite pools, such as aspartate, asparagine, glutamate, and serine [17]. These alterations in amino acid pools have not been investigated in humans. On the other hand, Regalis® that is formulated from prohexadione has been reported to bind and inhibit the Jmjd2a histone lysine demethylases activity *in vitro*. Consequently, this epigenetic modulation influences growth and differentiation of mouse nervous system (neurospheres) *in vitro* [18]. This mechanism could account for the toxicity that we observed on zebra fish larvae at 0.3 mg/mL Regalis®. For this product, we began to observe effects on the larvae at 0,1 mg/mL, a concentration similar to the EC50 measured on the rainbow trout *Oncorhynchus mykiss* using a different test (see Table 1). It is noteworthy that 1 mg/mL LBG-01F34® did not induced significant death in our *Danio rerio* assay, while this concentration is ten times the EC50 found in the rainbow trout test (Table 1).

Contrary to Regalis® and LBG-01F34®, BION-50 WG® had a pro-inflammatory effect on PBMC, inducing an overproduction of IL-1β whatever the dose tested. According to the manufacturer, BION-50 WG® contains 10–20 % silica to increase ASM penetration in plant leaves. Given that silica have known pro-inflammatory effects [19], it is possible that silica remaining in the supernatants that was used for the *in vitro* test might account for the up-regulated production of IL-1β in PBMC. However, these silica remains should be lower than the actual

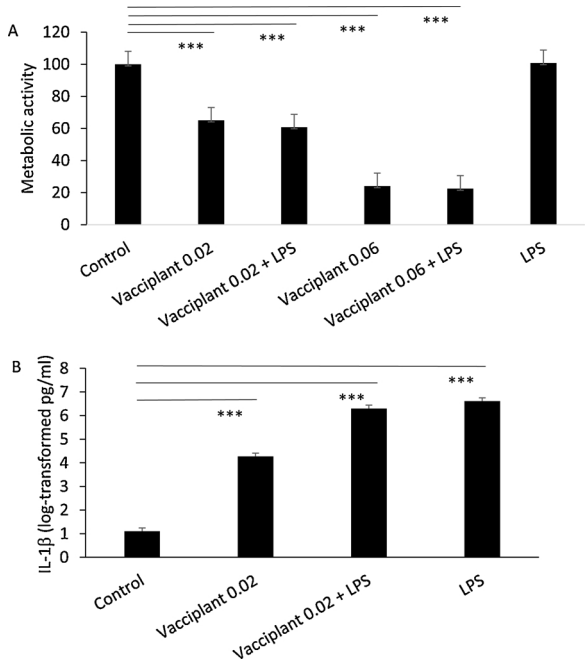

Fig. 4. Least squares means (+ SE) of metabolic activity (A) and IL-1β production (B) of human PBMC exposed to two doses of Vacciplant® (0.02, 0.06 mg/mL) alone or with LPS. Least squares means of metabolic activity were computed from a linear mixed effect model where the experimental group and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Least squares means of IL-1β production were computed from a linear mixed effect model where the experimental group (only the 0.02 mg/mL dose was tested here), metabolic activity and the replicate within the experimental group were the fixed effects and the identity of the blood donor the random variable. Metabolic activity is expressed as percent changes with respect to the control group, and IL-1β as log-transformed pg/mL.
Only statistically significant differences between the control and the treatment groups are indicated. The full details of all pairwise comparisons are reported in the online table S7 and S8.
*: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.

amount to which farmers are exposed to. When tested on zebra fish larvae, this product began to disturb development at 2.5 mg/mL, a concentration twice those of EC50 in the test using rainbow trout.

Vacciplant® had a very strong effect on PBMC metabolic activity measured by the XTT assay. At the dose of 0.02 mg/mL, metabolic activity was reduced by 35 % and at the dose of 0.06 mg/mL this reduction attained 76 %. Since XTT is classically used as a viability/proliferation test, our results suggest a cytotoxic effect of Vacciplant®. This is confirmed by estimation of PBMC viability when they were treated with 0.03 mg/mL Vacciplant® by Trypan blue. It corresponded to only 8% of cells while 92 % alive cells were found in controls (data not shown) and confirms the toxicity at this concentration. Vacciplant® also induced an overproduction of IL-1β. Vacciplant® is manufactured from laminarin, that is known to modulate animal innate immunity and to induce TNF-α and IL-6 mRNA in murine cultured cells [20]. It also inhibits phagocytosis in human macrophages [21]. Although the specific laminarin used in this PPP could be safe by itself, the action of β-glucans greatly depends on their molecular weight and conformation [22]. Furthermore, it is possible that excipients contained in Vacciplant® could be the cause of the observed effects on PBMC metabolic activity and IL-1β production. When tested on zebra fish larvae, this product began to disturb development at 2 µg/mL and was lethal at 20 µg/mL. These concentrations are much lower than the EC50 described for the rainbow trout assay (Table 1, 88 µg/mL). Furthermore, data of toxicity on zebra fish larvae using the same range of concentration as used for PBMC treatment clearly demonstrate that Vacciplant appears to be the most toxic of the 4 compounds tested.

Table 4
Effect of a 24 -h exposure to different concentrations of LBG-01F34, Regalis, Bion 50WG and Vacciplant on the mortality of 4-day-old zebra fish. The table reports the product tested, the different concentrations of active substance in water, the mean mortality with standard deviation, the number of replicates (for each replicate 20 zebra fish larvae were used), and a Kruskal-Wallis test to investigate whether mortality significantly differed among product concentrations.

Formulated product	Concentration (mg/ml)	Mean mortality % (sd)	Number of replicates	Kruskal-Wallis test (p value)
LBG-01F34	0	0 (0)	6	$\chi^2_4 = 5.00$ (0.2873)
	0.03	0 (0)	5	
	0.1	0 (0)	5	
	0.3	0 (0)	4	
	1	2.5 (5.0)	4	
Regalis	0	0 (0)	6	$\chi^2_3 = 16.23$ (0.001)
	0.1	3.75 (2.5)	4	
	0.3	100 (0)	4	
	1	100 (0)	4	
Bion 50WG	0	0 (0)	6	$\chi^2_4 = 17.4$ (0.0016)
	0.1	0 (0)	6	
	0.3	0.8 (2.0)	6	
	1	0 (0)	6	
	2.5	6.0 (4.2)	5	
Vacciplant	0	0 (0)	6	$\chi^2_3 = 24.86$ (< 0.0001)
	0.00025	0 (0)	10	
	0.002	30.0 (46.0)	6	
	0.02	100 (0)	6	

5. Conclusions

Since IL-1β is a key inflammatory cytokine, measuring its level is relevant to assess the potential toxicity of PPP. We found that formulated compounds can have inflammatory effects that were not described for molecules corresponding to their active compounds, probably because they were not investigated. Furthermore, it appears that toxicological tests must be conducted on the commercially available products, and not only on the active compounds, in order to avoid possible undesirable effects of the adjuvants [23]. These tests, fitting with the goal of reducing studies on animal models, could be routinely done to screen the potential adverse effects of compounds that can potentially cross-react with human innate immunity.

Declaration of Competing Interest

There are no conflicts of interest to declare.

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