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Evaluation of *Streptomyces* Common Scab Toxins Diffusion in Potato Tubers and through the Intestinal Barrier

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A B S T R A C T

K e y w o r d s Potatos, *Streptomyces scabies*, Thaxtomin A, Nigericin, Bafilomycin A1, Caco2/TC7 cells. **Article Info** *Accepted:* 17 April 2017 *Available Online:* 10 May 2017	*Streptomyces* and particularly *Streptomyces scabies* the causative agent of common scab in potato tubers are producing different toxins, including thaxtomin A, nigericin, geldanamycin, tuncamycin and bafilomycin. Assay of these toxins in two potato strains from the commercial circuit revealed that thaxtomin A can be detected in significant amounts in scab surface lesions but also in the flesh of the tubers. Other toxins were present under the detection limit of the assay. As thaxtomin A and other *Streptomyces* toxins are thermostable, potato consumers can be exposed to these molecules. In order to evaluate the potential effect of *Streptomyces* toxins on the intestinal barrier, their cytotoxicity was studied *in-vitro* on Caco2/TC7 enterocytes. Except geldanamycin, all other tested toxins showed significant cytotoxicity. Used at sub-lethal concentrations, *Streptomyces* toxins were without effect on the transepithelial resistance of a model of intestinal epithelium made by fully differentiated Caco2/TC7 cells cultured in inserts. However, measurements of the flow of these toxins through reconstructed intestinal epithelium suggest that thaxtomin A, geldanamycin and tuncamycin could cross the intestinal barrier. These results should deserve *in-vivo* studies to evaluate the real threat of these toxins in regard of consumers.

Introduction

Streptomyces are well known filamentous Gram-positive bacteria, essentially present in soil and considered as non-pathogen for humans (Kämpfer, 2006). They produce a large diversity of bioactive molecules, including antibiotics, antifungal and even immuno-modulators and are of major industrial interest (Watve *et al.,* 2001). However, several species of *Streptomyces* are phytopathogens and the first identified, *Streptomyces scabies,* is the principal responsible for potato common scab. This microorganism and related species are forming superficial lesions at the surface of potato tubers which affect both their production and commercial value. Because of the high frequency of this disease, reaching 85% for stocks in a survey realized in England (Dehnen-Schmutz *et al.,* 2010), and because it is considered having only superficial effects, potato tubers affected by common scab are entering the commercial

circuit and are consumed. This is not without raising some concerns as *S. scabies* and other *Streptomyces* involved in common scab are producing a large series of toxins, including thaxtomins, concanamycin, valinomycin, streptozotocin, bafilomycin, antimycins or nigericin (Bignell *et al*., 2014), which for the majority are encoded by a large pathogenicity island easily transferred between pathogenic and non-pathogenic strains (Kers *et al*., 2005; Lerat *et al*., 2009). Moreover, these toxins are heath stable and as *Streptomyces* hyphae are spreading deeply into the tuber (Loria *et al*., 2003), it is likely that these toxins can be found in cooked potato, particularly in products such as potato chips which are frequently presenting black spots relevant of *Streptomyces* infections (Myers *et al*., 2002; Knip *et al*., 2010). This is of potential importance as *Streptomyces* toxins, including thaxtomin A, nigericin, geldanamycin, tuncamycin and bafilomycin are suspected to be involved in acute forms of Type I diabetes (Myers *et al*., 2001; Myers *et al*., 2003; Bodin *et al*., 2015) and in chronic intestinal bowel diseases, including Crohn disease (Lowe *et al*., 2008).

In the present study, we investigated the distribution of these *Streptomyces* toxins in two types of potato tubers available in mass-market retailing. In complement, we used an *in vitro* model of differentiated enterocytes to document the potential intestinal resorption of these toxins.

Materials and Methods

Chemicals

Thaxtomin A, nigericin, tunicamycin, geldanamycin and bafilomycin A1 are presented in figure 1 with indication of their principal producing microorganisms. These toxins were obtained from Sigma Aldrich (Saint Quentin Fallavier, France). Ethanol,

dimethyl sulfoxide (DMSO), acetone and diethyl oxide were from the same provider.

Toxins assays in potato tubers

Toxins were assayed in two common varieties of potato tubers, one of small size (*Ratte du Touquet*, mean weigh 72g) and one of medium size (*Bintje*, mean weigh 170 g). They were obtained from a local commercial center. All potato tubers showed surface lesions typical of common scab. These surface lesions were collected for toxins assays. The flesh of the potato was also collected beneath the surface (0.5 cm and 1 cm under the surface for small and medium size varieties, respectively) and at the centre of the tubers. Each sample was mixed with the solvent required for toxin extraction (Ethanol for thaxtomin A and nigericin, DMSO for tunicamycin and geldanamycin) at a ratio of 1 mL solvent / 100 µg potato flesh. Samples were submitted to solvent extraction under gentle agitation (15 rpm) over 24 h at room temperature. Before analysis insoluble elements were discarded by centrifugation. Toxins were quantified using a SCIEX API 3000 LCMS-MS system (Applied Biosystems) equipped with an electrospray ionization interface used to generate positive ions [M+H]+.

The analytes were separated on reversed phase column (Waters symmetry C18, 50 x 2.1 mm; 3.5 µ particle size) with a gradient of mobile phase. Mobile phase A consisted in 20 mM ammonium acetate in water and mobile phase B of 0.1% of formic acid in acetonitrile. The following gradient was used: 0 to 1.5 min isocratic A/B 85%A/15%B; 1.5 to 2.0 min to reach 20%A/80%B maintained until 4.0 min, return to 85%A/15%B in 0.1 min and stay isocratic during 2.9 min. The column and autosampler temperature were maintained at 40°C and room temperature respectively. The mobile phase was eluted at 0.5 mL/min using

a Perkin Elmer 200 system. The optimized ion spray voltage and temperature were set at 5500 V and 500 °C. The typical ion source parameters, declustering potential (DP), collision energy (CE), entrance potential (EP) and collision cell exit (CXP) potential were 50, 32, 9 and 20 V for thaxtomin A; 50, 60, 10 and 15 V for nigericin; 30, 30, 8 and 15 V for geldanamycin; 60, 28, 12 and 10 V for tunicamycin. Nitrogen gas was used for the nebulizer gas, curtain gas and collision-activated dissociation gas, which were set at 10, 15 and 3 psi respectively. Quantification was performed by selected reaction monitoring of the protonated related product ion using the internal standard (IS) method with peak area ratios and a linear least-squares regression curve with a weighting factor of $1/x2$. The internal standard was geldanamycin for thaxtomin A, and geldanamycin for thaxtomin A, no internal standard were used for nigericin nor tunicamycin. The mass transition used for thaxtomin A, nigericin, geldanamycin, and tunicamycin were m/z 439.2 _ 247.0, 747.4 _ 729.6, 578.4 _ 468.3 and 817.4 _ 596.4 respectively, with a dwell time of 100 millisecond transition. Quadrupoles Q1 and Q3 were set on unit resolution. The analytical data were processed by analyst software (Version 1.4.1; Applied Biosystems).

Cytotoxicity assays

The human colonic adenocarcinoma cell line Caco-2/TC7 cells was employed to test the cytotoxicity of *Streptomyces* toxins and their solvents. These cells were used between passages 40-60. Caco-2/TC7 cells C were grown in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 15% heat inactivated fetal calf serum (FCS), 2 mM L-glutamine, 100 U.ml each of penicillin and streptomycin, and 1% non-essential amino acids. The cells were seeded and cultivated at 37°C in 5% CO_2-95% air

atmosphere, in 24 wells tissue culture plates until confluence for cytotoxicity assay and on inserts (6.4 mm diameter, 3 µm pore size, Falcon) until full differentiation (28 days) for measurement of transepithelial resistance and toxins flow and permeability.

Transepithelial resistance measurements

Transepithelial resistance (TER) was used to monitor Caco-2/TC7 differentiation and confluence on inserts when the cells were grown in the absence or presence of *Streptomyces* toxins. Toxins were tested at concentrations close to their NOEL values determined in cytotoxicity assays. TER was measured at days 7, 21, 23, 25 and 28 using a Millicell Electrical Resistance System (Millipore Corp, Bedford, MA). The minimal TER value corresponding to confluent and fully differentiated Caco2/TC7 cells forming a continuous pseudo epithelium was 930 Ω.

Measure of toxin flow and permeability

Transepithelial permeation was used to evaluate the potential of *Streptomyces* toxins to cross the intestinal barrier. This parameter was measured using fully differentiated Caco2/TC7 cells grown after 28 days of culture on inserts (6.4 mm diameter, 3 µm pore size, Falcon) with 350 µL DMEM in the apical compartment and 900 µL in the basolateral compartment. The flow was calculated on the basis of the toxin concentration measured in the basolateral compartment after 24 h, as previously described, and considering the volume of this compartment (900 µL) and the surface of the insert (0.3 cm^2). Permeability was obtained by division of the flow value by 3600 and by the toxin concentration used in the apical compartment. The amount of toxins measured in the apical and basolateral compartments after 24 h incubation was compared to the amount inoculated at the beginning of the

experiment to evaluate any potential degradation or adsorption of tested molecules.

Statistical analysis

All results are expressed as means ± standard error (SEM) calculated over a minimum of three independent experiments. Statistical differences were estimated using the Student's *t* test and were noted as★, ★★ and ★★★ for *p*-values< *0.05*,< *0.01* and< *0.001*, respectively. In confocal microscopy studies, the thickness of the biofilms was calculated from a minimum of 20 measures in different fields.

Results and Discussion

Cytotoxic activity of solvents required for *Streptomyces* toxins solubilisation

Thaxtomin A, nigericin, tunicamycin, geldanamycin and bafilomycin A1 can be only solubilised by solvents such as ethanol, DMSO, Diethyl ether or acetone. Then, in order to further investigate the activity of *Streptomyces* toxins on Caco2/TC7 cells, it was necessary to control the cytotoxicity of these solvents. As shown in figure 2 when they were used at a final concentration under 1% these solvent had no or very limited cytotoxicity on Caco2/TC7 cells. Considering their optimal solubility, ethanol 1% was selected for solubilisation of thaxtomin A and nigericin in DMEM and DMSO 1% for solubilisation of tunicamycin, geldanamycin and bafilomycine A1.

Assay of *Streptomyces* toxins in potato tubers

The same solvents were used to extract each *Streptomyces* toxin from potato tubers. Toxins were assayed in scab surface lesions, in the flesh beneath the surface and at the centre of the tubers (Fig. 3). In the small variety of

potato tubers (mean weight 72 g), the concentration of thaxtomin A measured in surface lesions reached 3.3 ng / 100 µg of tissues. At a depth of 0.5 cm, this mean concentration of thaxtomin A decreased to 2.93 ng / 100 µg but remained of 2.29 ng / 100 µg at the centre of the tubers. In the larger variety (mean weight 170 g), the concentration of thaxtomin A decreased from 1.99 ± xx ng / 100 µg in surface lesions to 0.63 ng / 100 µg at the centre. Nevertheless, these measures reveal that significant amounts of the thaxtomin A are present in the flesh of potato tubers affected by common scab. Conversely, all other toxins were under the detection limits, i.e. < 5 ng / 100 µg for geldanamycin and < 10 ng / 100 µg for nigericin and tunicamycin. Bafilomycin was not assayed.

Cytotoxic activity of *Streptomyces* toxins on Caco2/TC7 cells

The cytotoxic activity of *Streptomyces* toxins was measured using the LDH assay and was compared to the basal level of cell death measured in control untreated cells (15 ± 1 %) and to the maximal cell death (100 %) induced by administration of pure solvents (ethanol or DMSO). Thaxtomin A 100 µg/mL had a significant cytotoxic activity on Caco2/TC7 cells with a mean cell death of 25.1 ± 2 %, whereas lower doses were not inducing any significant increase of cytotoxicity. Nigericin 50 µg/mL induced the death of 100 % of Caco2/TC7 cells. At 5 µg/mL the cytotoxicity of nigericin remained high (58.8 ± 6 %) but it decreased to 20.2 ± 3 % when it was administered at a dose of 500 ng/mL. Even at the highest concentration tested (100 µg/mL) the cytotoxicity of geldanamycin remained in the same range as the control (16.7 ± 1 %). Tunicamycin 100 µg/mL was leading to the death of all Caco2/TC7 cells. When its concentration was reduced to 10µg/mL the cytoxicity of

tunicamycin decreased to 22 ±1 % and to 16.5 ± 2 % when it was administered at a concentration of 1 µg/mL. Bafilomycin A1 1µg/mL was associated to the death of 43.4 ± 4 % of the cells. Lower concentrations allowed to reduce its cytotoxicity but even at the lower dose tested (1 ng/mL) it remained significantly higher than the control (23.6 ± 1 %) (Fig. 4).

Effect of sub-lethal concentrations of *Streptomyces* toxins on Caco2/TC7 cells differentiation

Toxins were tested at concentrations close to their NOEL values on Caco2/TC7 cells as determined previously: 10 µg/mL for thaxtomin A, 500 ng/mL for nigericin, 100 µg/mL for geldanamycin and 1 µg/mL for tunicamycin. Bafilomycin A1, being toxic even at the lower dose administered, was not tested. As previously observed, complete differentiation of Caco2/TC7 cells was not achieved before 28 days of culture on inserts (Fig. 5). TER measured after cell growth in the presence of low doses of solvents (ethanol or DMSO 1%) required to solubilize toxins was higher suggesting that cells adapted to solvents by reducing their membrane permeability. None of the toxins tested was decreasing the TER of cells monolayers after 28 days of cultures in their presence indicatig that, when employed at sublethal doses thaxtomin A, nigericin, geldanamycin and tunicamycin are not affecting the differentiation of Caco2/TC7 cells.

Measure of toxin flow and permeability of *Streptomyces* toxins through fully differentiated Caco2/TC7 cells monolayers

Thaxtomin A was presenting a significant flow (2.83 ± 0.34 ng/h/cm2, Fig. 6A) and permeability (2.91 ± 0.4 cm/s, Fig. 6B) on differentiated Caco2/TC7 epithelium after 24 h incubation on inserts. The cumulated mass

of thaxtomin A recovered in the apical and basolateral compartments of inserts was lower (- 13.9 ± 1.2 %) than the initial mass of the toxin layered at the onset of the experiment suggesting a partial degradation or adsorption of the toxin on the cells. Nevertheless, the values measured are consistent with a real flow of thaxtomin through the Caco2/TC7 cells epithelium. Nigericin was not detected in the basolateral compartment of inserts after 24 h incubation and the apparent resulting flow and permeability were null. However, nigericin was also undetectable in the apical compartment indicating that in our experimental conditions this toxin was not sufficiently stable for measuring its flow and permeability. In the case of geldanamycin and tunicamycin a marked difference was also observed between the amount of the toxin initially administered and that recovered in total after 24 h (- 67,0 ± 4.7 % and - 76.5 ± 9.8 %, respectively) suggesting that these toxins were also adsorbing or were actively degraded by Caco2/TC7 cells. However, flow (0.75 ± 0.1ng/h/cm2 and 0.09 ± 0.18 ng/h/cm2 for geldanamycin and tunicamycin, respectively) and permeability values (0.531 ± 0.2 cm/s and 12.1 ± 0.5 cm/s for geldanamycin and tunicamycin, respectively) remained usable indicating that both toxins were capable to cross the Caco2/TC7 epithelial model.

Food safety is a major concern, especially for products of common consumption which can lead to important cumulative amounts of toxins in case of contamination. This is potentially the case of potato with a mean consumption of 60 and 87.5 kg/capita in North America and Europe (FAOSTAT, 2008). Indeed, potatoes are very frequently affected by a disease caused by different species of *Streptomyces* designated as "common scab". This disease can lead to important production losses (Hill and Lazarovits, 2005) and tubers showing marks

of common scab are frequently found in the commercial circuit. As in its mild form this disease affects apparently only the surface of the tuber, they can be easily consumed. However, *Streptomyces* hyphae might penetrate beneath the surface of the tubers (Clark & Matthews, 1987; Lauria *et al*., 2003) and most of the toxins produced by these microorganisms are thermostable. Then, consumers should be exposed to these toxins through cooked or transformed potato products.

In the present work we investigated the distribution of thaxtomin A, nigericin, tunicamycin, geldanamycin and bafilomycin A1, five toxins produced by *S. scabies*, the principal germ responsible for common scab, and related species. As those toxins are not soluble in water or cell culture medium, we selected two solvents, *i.e.* ethanol and DMSO 1%, allowing to solubilize and extract toxins from potato flesh and without intrinsic toxicity that should interfere with the effect of *Streptomyces* toxins on Caco2/TC7 cells. The colon carcinoma Caco2 cell line is the only human epithelial cell line which differentiates spontaneously into enterocytes when grown in standard culture conditions including formation of microvilli and specific enzymatic activities at the apical surface (Turk *et al*., 2004) and provides a simplified model to investigate intestinal cells differentiation and permeability.

Assay of the toxins in two varieties of potato tubers collected randomly in the commercial circuit revealed that thaxtomin A is not only localized in surface scab lesions, but can be detected in significant amounts beneath the surface and just to the centre of the tuber. Other toxins studied, *i.e.* nigericin, tunicamycin, geldanamycin and bafilomycin A1, were present at concentrations below the detection limits of our analytical tools. However, this is not excluding a potential

effect of these molecules as additive or even synergistic effects of these compounds have been described (Hiltunen *et al*., 2006; Duke & Dayan, 2011). Even when studied independently, most of these molecules showed significant toxicity on Caco2/TC7 cells. Thaxtomin A 100 µg/mL induced a significant increase of cell death but reduced concentrations under 10 µg/mL were without effect. The cytotoxicity of nigericin was high (100 % cell death at 50 µg/mL) and it was necessary to reduce the concentration to 500 ng/mL to get closer to the NOEL value. Tunicamycin also showed high toxicity and its NOEL value was in the same range (1 µg/mL). Geldanamycin was the only molecule which did not show any toxicity in our experimental conditions, even at the highest dose tested (100 µg/mL). Conversely, the toxicity of bafilomycin A1 remained significant at all tested doses, including the lower (1 ng/mL), The toxicity of Thaxtomin A and other thaxtomins has been almost exclusively investigated in vegetals where these molecules cause cell hypertrophy and apoptosis (King & Calhoun, 2009). To our knowledge, until now the unique study on the toxicity of thaxtomins in animals was realized on spermatozoids where no acute effect on mobility was observed (Kotiaho *et al*., 2008) but other potential effects were not studied. Nigericin is a teratogenic compound (Vedel-Macrander & Hood, 1986) and its toxicity (LD50) is reaching 2.5mg/kg by parenteral administration in mouse (Harned *et al*., 1951). This is coherent with the high toxicity of this molecule on Caco2/TC7 cells. Tunicamycin is showing apoptotic activity and its toxicity on developing cells has been also well documented (Balcan & Arslan, 2015). Geldanamycin is an inhibitor of the chaperone protein Hsp90 (Han *et al.,* 2014) with apoptotic activity. In this regard its total absence of acute toxicity on Caco2/TC7 cells was unexpected.
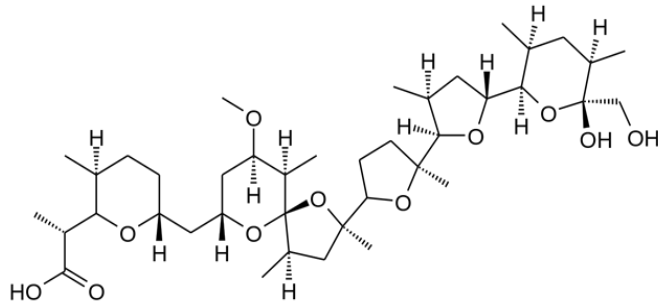
Fig.1 Structure of the more abundant toxins produced by *Streptomyces scabies* and related species
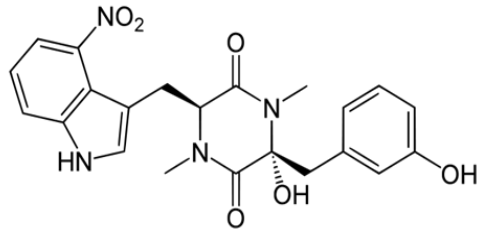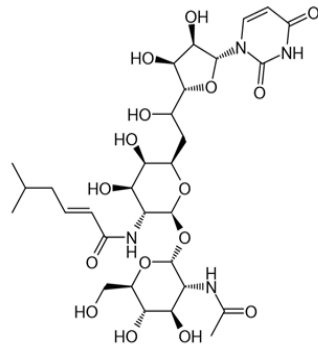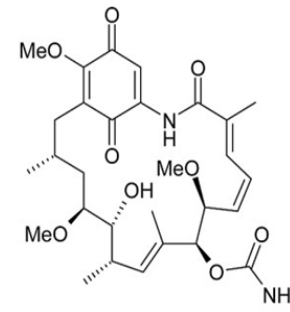
Thaxtomin A (+++ *S. scabies*)
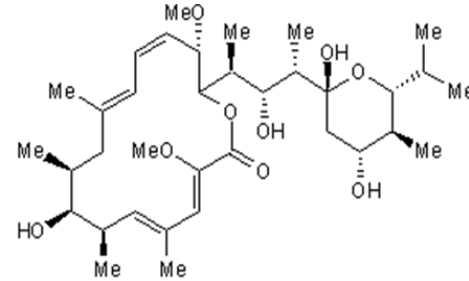
Bafilomycin A1 (+++ *S. griseus, S. scabies*)

Nigericin (+++ *S. hygroscopicus, S. scabies*)

Geldanamycin (+++ *S. hygroscopicus*)

Tunicamycin (+++ *S. lysosuperificus*)

Fig.2 Cytotoxic activity of solvents required for *Streptomyces* toxins solubilisation
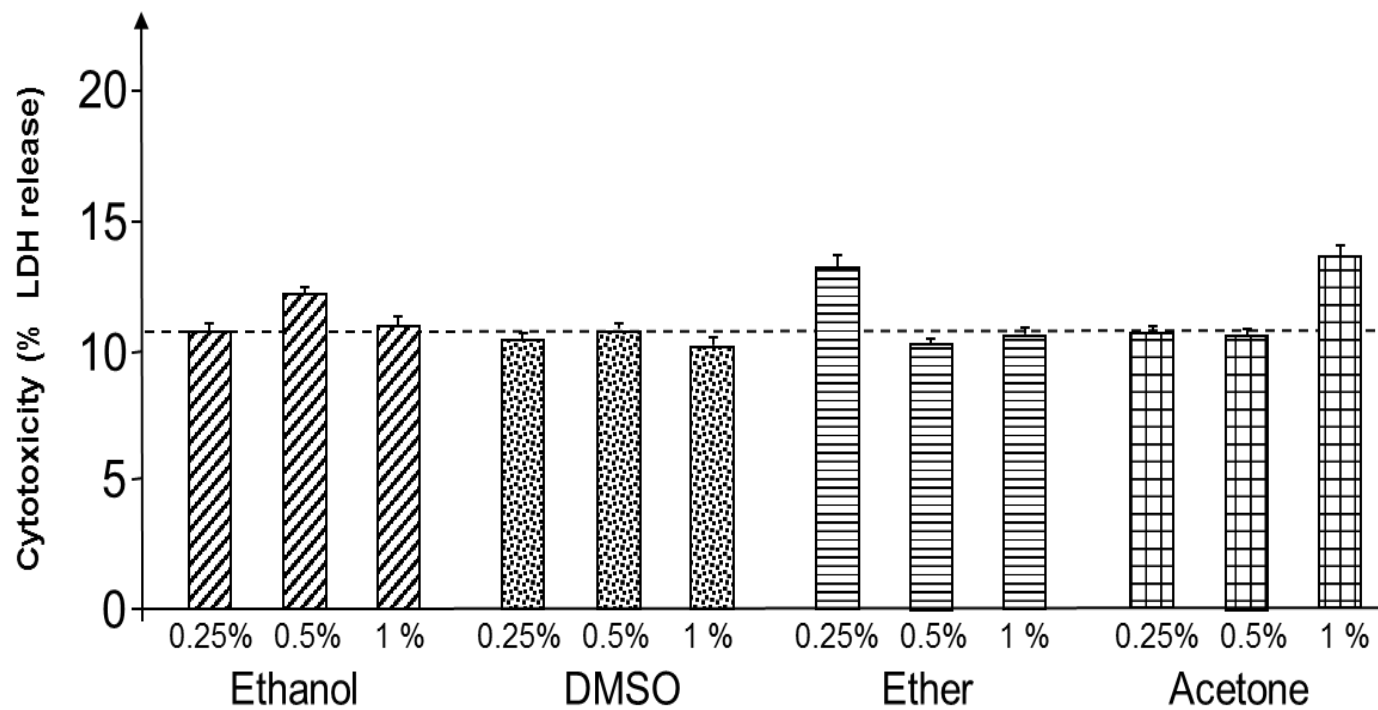on CaCo2/TC7 cells. (★★ = p < 0.01; ★★★ = p < 0.001)

Fig.3 Concentrations of Thaxtomin A, Nigericin, Geldanamycin and Tunicamycin measured in common scab lesions, sub-peripheral areas and at the center of two varieties of potato tubers
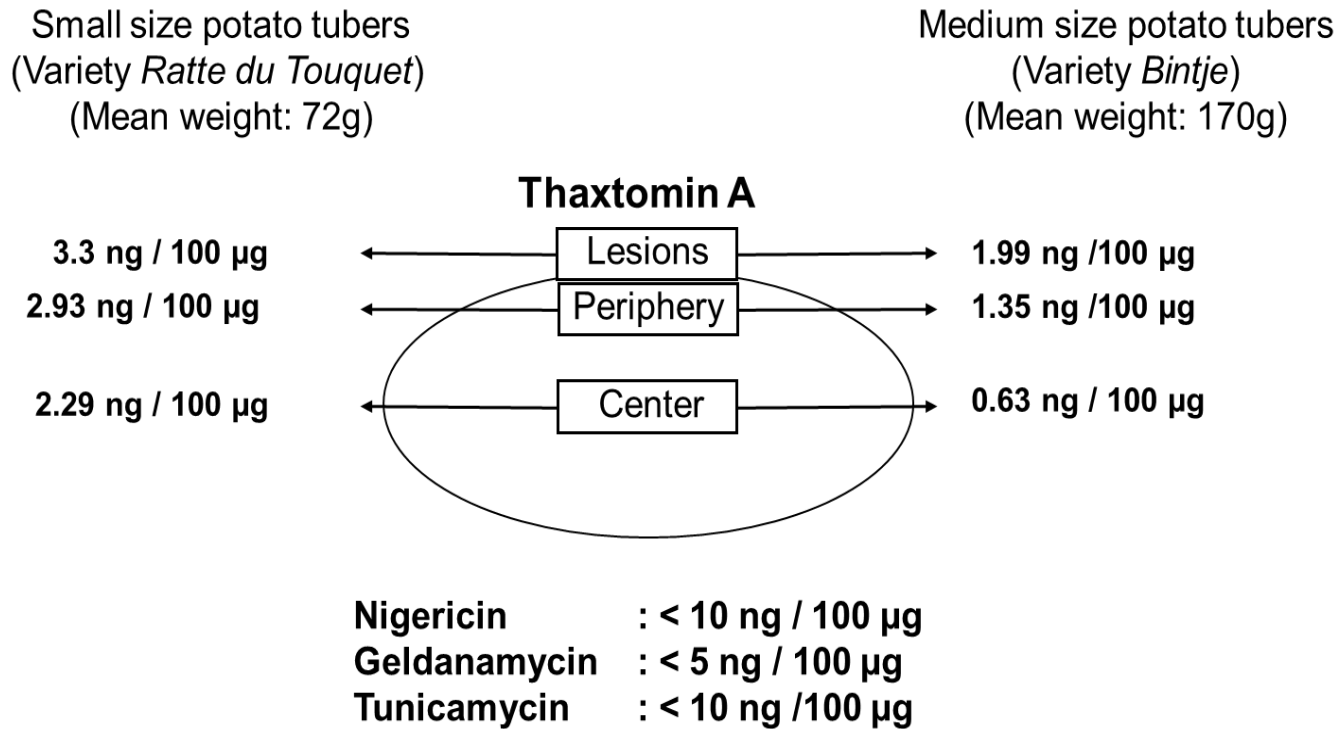
Small size potato tubers
(Variety *Ratte du Touquet*)
(Mean weight: 72g)

Medium size potato tubers
(Variety *Bintje*)
(Mean weight: 170g)

Thaxtomin A

3.3 ng / 100 µg	Lesions	1.99 ng /100 µg
2.93 ng / 100 µg	Periphery	1.35 ng /100 µg
2.29 ng / 100 µg	Center	0.63 ng / 100 µg

Nigericin : < 10 ng / 100 µg
Geldanamycin : < 5 ng / 100 µg
Tunicamycin : < 10 ng /100 µg

Fig.4 Cytotoxic activity of Thaxtomin A, Nigericin, Geldanamycin, Tunicamycin and Bafilomycin A1 on CaCo2/TC7 cells. (★★ = p < 0.01; ★★★ = p < 0.001)
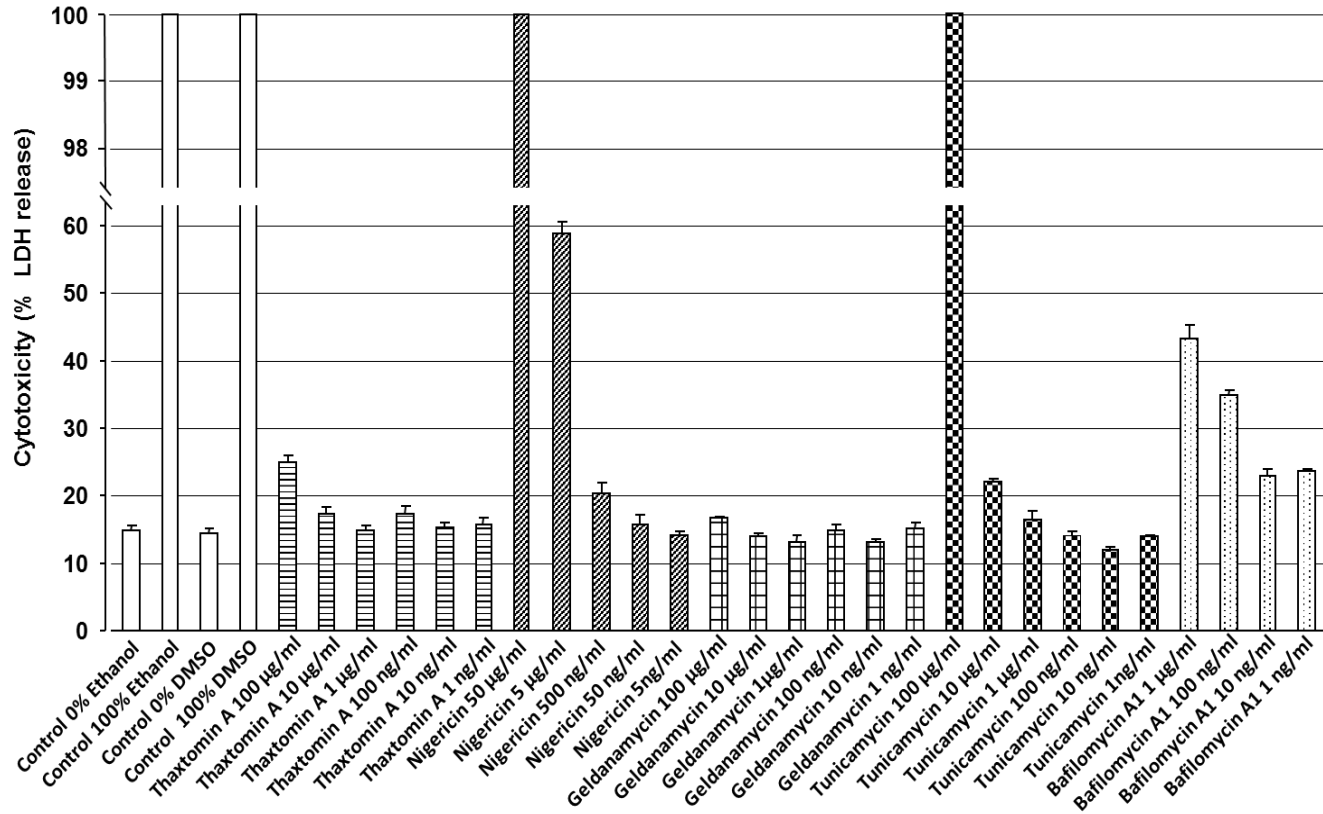
Fig.5 Effect of Thaxtomin A, Nigericin, Geldanamycin and Tunicamycin on the trans-epithelial resistance of CaCo2/TC7 monolayers during differentiation over 28 days
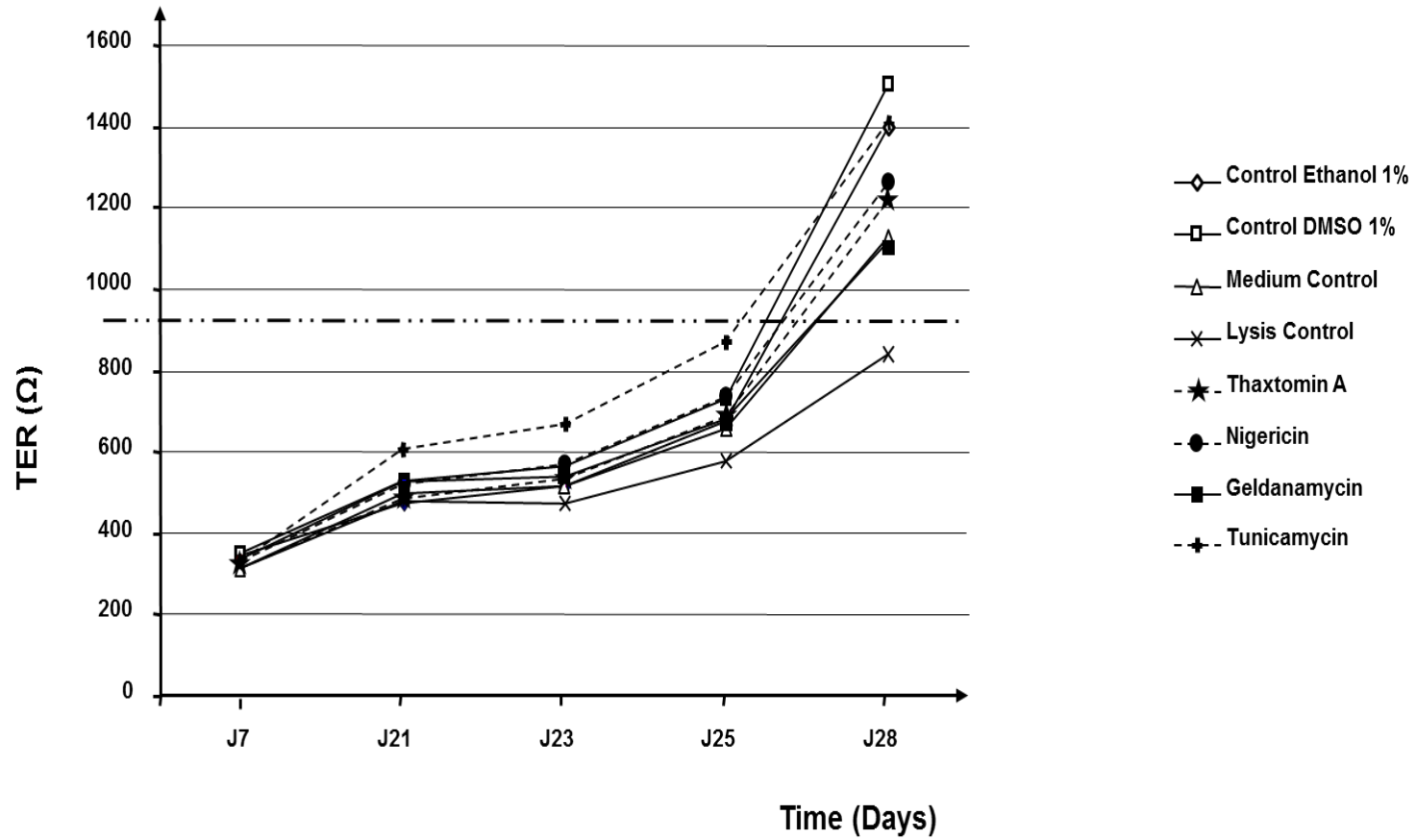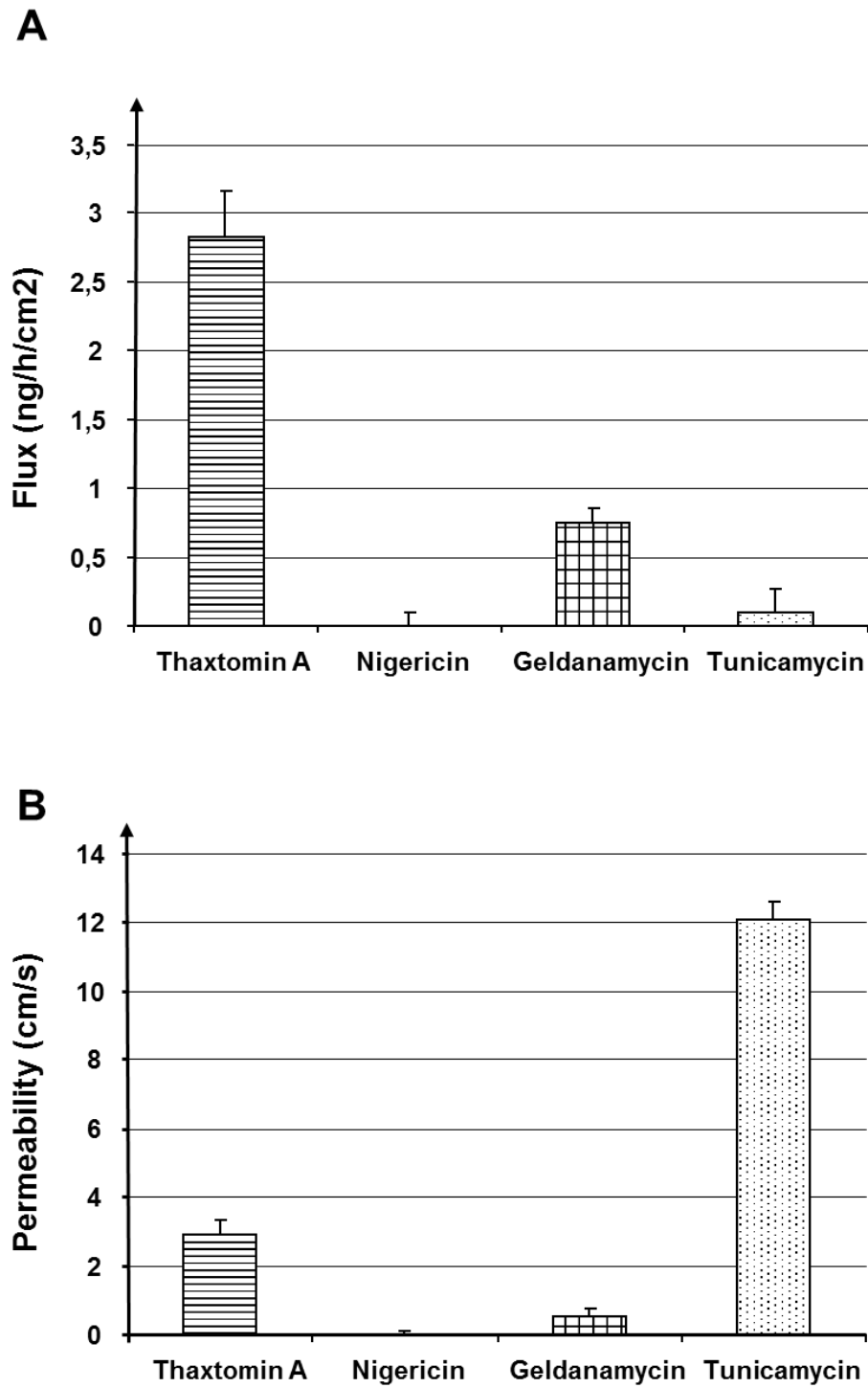
Fig.6 Mean flux (A) and permeability (B) of Thaxtomin A, Nigericin, Geldanamycin and Tunicamycin through fully differenciated Caco2/TC7 intestinal like epithelium. (NS = non significant; ★ = p < 0.05; ★★ = p < 0.01; ★★★ = p < 0.001)

Conversely, the cytotoxicity of bafilomycin A1 is coherent with its strong apoptotic activity (Kinoshita *et al*., 1996). In order to avoid any acute effect and reflect the conditions of chronic exposure to low doses of these toxins, their effect on Caco2/TC7 cells differentiation was investigated in the range of their NOEL values, *i.e.* 10µg/mL for thaxtomin A, 500 ng/mL for nigerin and 1 µg/mL for tunicamycin. Geldanamycin was used at the maximal dose tested in cytotoxicity studies (100 µg/mL) and bafilomycin A1 was excluded from the rest of the study because of its significant toxicity at alltested doses. Caco2/TC7 cells were exposed to the toxins over 28 days all along their differentiation into enterocytes and assembly into an epithelium. Differentiation was monitored by measuring the evolution of TER. The TER value of tunicamycin treated cells showed a marginal increase between J7 and J21 in comparison to the control and all other tested conditions. However, after 28 days of culture, the TER value of all epithelia was above 930 Ω indicating that they were fully differentiated and that, at the sub-lethal values employed, none of the toxins studied had a significant effect of the differentiation of Caco2/TC7 cells. In this condition it was possible to investigate the flux of these toxins through the differentiated epithelia.

Despite a partial loss due to adsorption or degradation a significant flow of thaxtomin A, geldanamycin and tunicamycin was measured through the reconstructed enterocyte epithelium. Thaxtomin A was presenting a significant flow (2.83 ± 0.34 ng/h/cm2, Fig. 6A) and permeability (2.91 ± 0.4 cm/s, Fig. 6B) on differentiated Caco2/TC7 epithelium after 24 h incubation on inserts. The cumulated mass of thaxtomin A recovered in the apical and basolateral compartments of inserts was lower (- 13.9 ± 1.2 %) than the initial mass of the toxin layered at the onset of the experiment suggesting a partial degradation or adsorption of the toxin on the cells. Nevertheless, the values measured are consistent with a real flow of thaxtomin through the Caco2/TC7 cells epithelium.

Nigericin was not detected in the basolateral compartement of inserts after 24 h incubation and the apparent resulting flow and permeability were null. However, nigericin was also undetectable in the apical compartment indicating that in our experimental conditions this toxin was not sufficiently stable for measuring its flow and permeability. In the case of geldanamycin and tunicamycin a marked difference was also observed between the amount of the toxin initially administered and that recovered in total after 24 h (– $67,0 \pm 4.7$ % and - 76.5 ± 9.8 %, respectively) suggesting that these toxins were also adsorbing or were actively degraded by Caco2/TC7 cells. However, the flow (0.75 ± 0.1 ng/h/cm2 and 0.09 ± 0.18 ng/h/cm2 for geldanamycin and tunicamycin, respectively) and permeability values (0.531 ± 0.2 cm/s and 12.1 ± 0.5 cm/s for geldanamycin and tunicamycin, respectively) remained usable, indicating that both toxins were capable to cross the Caco2/TC7 epithelial model.

In conclusion, this study reveals that in potato affected by common scab a toxin such as thaxtomin A diffuses deeply just to the center of the tuber. Although other studied toxins were below the detection limit, this is indicating that *Streptomyces* toxins can be potentially present in the whole flesh of infected tubers. These toxins are heath stable, and are presenting significant cytotoxicity on Caco2/TC7 enterocytes but appear having a limited impact on the intestinal barrier. In contrast, *in vitro* studies suggest that even at sub-lethal doses thaxtomin A, geladanamycin and tunicamycin can cross the intestinal epithelium. The risk of acute toxicity is very limited but chronic exposure to this toxin could lead to sensitization. The present results

deserve an *in vivo* evaluation of the risk associated to *Streptomyces* toxins.

Acknowledgements

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