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Detached leaf *in vitro* model for masked mycotoxin biosynthesis and subsequent analysis of unknown conjugates

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Abstract

The manuscript details the development of an *in vitro* model plant system using detached leaves because there is a need for biosynthetic methods for the production and isolation of masked mycotoxins. This detached leaf *in vitro* model was firstly applied to deoxynivalenol with satisfying results. The biosynthesis of deoxynivalenol-3-glucoside was confirmed using its respective commercially available reference standard. The detached leaf *in vitro* model was also secondly applied to T-2 toxin. Mono- and tri-glucoside derivatives of T-2 toxin and HT-2 toxin, T-2-(3)-glucoside, T-2-(3)-triglucoside and HT-2-(3)-glucoside were identified and characterized using Orbitrap high-resolution mass spectrometry. This is the first report on a triglucoside of T-2 toxin. The discovery of new masked forms implies the importance of the development of analytical methods for their detection, the constitution of toxicity studies, and proving the relevance of presence in the food and feed chain.

Keywords: untargeted analysis, high resolution mass spectrometry, Orbitrap, masked mycotoxins, detached leaf

1. Introduction

In recent years, scientific awareness of masked mycotoxins in food, feed and unprocessed grain samples has increased (Berthiller *et al.*, 2012; De Boevre *et al.*, 2012a; Skrbic *et al.*, 2011). Focus has traditionally been directed towards the glucosylated metabolite of the trichothecene mycotoxin deoxynivalenol (DON), namely deoxynivalenol-3-glucoside (DON-3G), however for other mycotoxins such as T-2 toxin (T-2) many derivatives remain unknown. Also, the unavailability of analytical reference standards makes it difficult to include masked conjugated mycotoxins in routine analytical methods.

Several strategies for masked mycotoxins' synthesis have been described. Chemical synthesis was delineated for conjugates of zearalenone (ZEA) using the Koenigs-Knorr reaction (Grabley *et al.*, 1992; Zill *et al.*, 1990). Also, DON-3G and deoxynivalenol-15-glucoside (DON-15G) were synthesized according to a modified protocol in a two-step reaction from 1-β-bromo-1-deoxy-2,3,4,6-tetra-*O*-acetyl-α-d-glucose and the acetylated forms of DON

(Savard, 1991). The execution of chemical synthesis offers a high yield, however, can be laborious and expensive. Another approach was a fermentation procedure for the production of zearalenone-14-glucoside (ZEA-14G), using an engineered *Saccharomyces cerevisiae* strain, expressing the *Arabidopsis thaliana* UDP-glucosyltransferase UGT73C6 (Berthiller *et al.,* 2006). In addition, the same results were observed for *Rhizopus* spp. (Kamimura, 1986). Recently, yeasts of *Trichomonascus clade* were used for the production of T-2 glucosides (McCormick *et al.,* 2012). Furthermore, the feeding of native toxins to model systems is promising as well. For example, McCormick *et al.* (2012) selected species of the *Trichomonascus* yeast as potential candidates for trichothecene modification because these were earlier found to metabolize compounds such as adenine, xanthine, glycine, uric acid, putrescine, and branched-chain aliphatic compounds (Middelhoven and Kurtzman, 2003). Three *Blastobotrys* species converted T-2 into T-2-(3)-glucoside (T-2-(3)G) (McCormick *et al.*, 2012).

In recent years, new methods for production of glucosylated mycotoxins have been developed in close association with molecular plant pathology studies. The glucosylation of DON to DON-3G constitutes a major resistance trait for the wheat plant (Lemmens *et al.*, 2005), a great deal of research has been dedicated to elucidating which genes are responsible for this transformation. These genes are of considerable importance to breeders, but their potential as sources for DON-3G and other glucosides for the analytical community should not be underestimated. Poppenberger *et al.* (2003) described the *Arabidopsis* gene AtUGT73C5 which effectively provides resistance against DON in yeast, but has undesired phenotypic side effects in mature plants during over-expression (Poppenberger *et al.,* 2003). Schweiger *et al.* (2010) identified a barley glucosyltransferase (HvUGT13248) conferring resistance against DON, which was then cloned into *Arabidopsis* and was shown to be capable of glucosylating significant amounts of DON in this model system (Schweiger *et al.,* 2010; Shin *et al.,* 2012).

Another natural approach is the treatment of cereals with mycotoxins or *Fusarium* strains. Treatment of cereals with DON (Berthiller *et al.,* 2005), or alternatively spray-inoculation with either *F. graminearum* or *F. sporotrichioides* at anthesis were described by Buerstmayr *et al.* (2004) and Busman *et al.* (2011), respectively (Buerstmayr *et al.,* 2004; Busman *et al.,* 2011). However, the glucoside extraction procedure from naturally or artificially contaminated grain samples is troublesome.

Current multi-mycotoxin methods are based on the separation and detection either by analysis of preselected compounds with triple quadrupole MS or by the untargeted detection in full scan mode with high resolution mass spectrometry (HRMS). With the latter method, the preselection of targeted compounds is no prerequisite and compound detection depends on the predefined full scan range (Zweigenbaum, 2011). Furthermore, the exploitation of full scan acquisition data to obtain additional information on the complex fungal metabolome has been investigated (Cirlini *et al.,* 2012).

In order to uncover the broadest range of mycotoxin conjugates as possible, an *in vitro* detached leaf model system was developed. This procedure stems from the notion that one-week old detached leaves in a fully controlled environment are able to metabolize mycotoxins, as adult plants can. In this study, a model system was developed to detect and partially characterize new conjugates of *Fusarium* mycotoxins in a fast set-up. In this model system, pure toxin was inserted to an *in vitro* set-up of detached leaves of wheat and seedlings of maize.

The European Commission has set maximum limits for DON in foodstuffs (1881/2006/EC, 2006) and guidance values for DON in products intended for animal feeding (2006/576/EC, 2006). Recently indicative values for the sum of T-2 and HT-2 were introduced according to Commission Recommendation of 27/03/2013 regarding the occurrence of T-2 and HT-2 in cereals and cereal products in food and feed (2013/165/EU, 2013). Masked mycotoxins have not yet been taken into account in these legislative bodies due to the lack of *in vivo* toxicological relevance of masked mycotoxins. Toxin choice fell to DON and T-2, for which legislation is in place. Recent reports on the occurrence of metabolites of T-2 describe the application of high resolution mass spectrometry via untargeted screening of samples (Busman *et al.*, 2011; De Angelis *et al.*, 2013).

Therefore, the analysis of possible metabolites of T-2 after an incubation step, using state of the art Orbitrap technology is discussed.

2. Materials and methods

Detached leaf *in vitro* model

Successful germination of the maize seeds (*Zea mays* 'Dominator', *Advanta*) was acquired through the incubation on an artificial germination medium. Before incubation, seeds were wrapped in filter paper, sterilized during 15 minutes in 0.75% NaOCl (local supermarket) and 25 µL L^{-1} Tween® 20 (Sigma Aldrich NV/SA, Belgium), and rinsed three times with deionized sterile water. Water was obtained from a Milli-Q® SP Reagent water system from Millipore Corp. (Brussels, Belgium). The germination medium was based on 20 mL solid Murashige and Skoog medium (Murashige, 1962), supplemented with 30 g L^{-1} sucrose (Sigma Aldrich NV/SA, Belgium) and 6 g L^{-1} fyto agar (Duchefa Biochemie, The Netherlands). The pH of the medium was adjusted to 5.8 ± 0.1 prior to autoclaving (121 °C, 15 min). The pH was measured with a HI 2250 pH meter (Hanna Instruments, Belgium) and adjusted with 1M NaOH (Sigma Aldrich NV/SA, Belgium) or HCl (Sigma Aldrich NV/SA, Belgium). Each seed was germinated in a glass tube (24 mm x 140 mm) during 5 days at 26 ± 2 °C under a 16-h photoperiod provided by cool white fluorescent lights (40 µE m^{-2} s^{-1}). When seedlings had a length of approximately 9 cm, a 3 cm stem part was cut and antiseptically transferred, per 4 parts, in a 15 mL polypropylene conical centrifuge tube (Falcon®, Genaxxon, Germany) filled with 10 mL of liquid Murashige and Skoog medium (Murashige, 1962). The medium was made by supplementing 30 g L^{-1} sucrose (Sigma Aldrich NV/SA, Belgium) with 0.6 g L^{-1} fyto agar (Duchefa Biochemie, The Netherlands). In order to maintain a liquid medium, the fyto agar was added in a ten times less ratio than the solid medium. The set-up of the detached leaf model is illustrated in **Figure 1**.
At this point, the parent toxins DON or T-2 (> 99% purity), diluted in DMSO/0.01N KOH (10/90, v/v) to concentrations of 1 mg L^{-1} and 10 mg L^{-1} were added to the medium and the tube was placed on a vertically shaker (Innova 4000, Artisan Scientific Corporation, Illinois, USA), at 28 °C under a 16-h photoperiod provided by cool white fluorescent lights (20 µE m^{-2} s^{-1}). Individual mycotoxin solid standards (25 mg) of DON and T-2 (molecular weight 296 and 466 g $mole^{-1}$, respectively) were obtained from Fermentek (Jerusalem, Israel). Potassium hydroxide (KOH) was obtained by Sigma Aldrich NV/SA (Bornem, Belgium). Dimethylsulfoxide (DMSO) was supplied from Labconsult (Brussels, Belgium). Deoxynivalenol-3-glucoside (DON-3G) (50.2 ng μL^{-1} in acetonitrile) is the only masked mycotoxin commercially available and was purchased by Biopure Referenzsubstanzen GmbH (Tulln, Austria). After 3 days, detached leaves were removed from the test tubes and the remaining medium was processed.

Detached leaf *in vitro* models with wheat were set up in a similar manner as with maize. The Chinese cultivar Sumai3 bearing amongst others the QTL Qfhs.ndsu-3BS was utilized, and incubation with parent mycotoxins lasted for 10 days. Mycotoxins DON and T-2 were investigated.

Sample preparation was based on the method described in De Boevre *et al.* (2012b) (De Boevre *et al.*, 2012b), however minor adjustments were introduced. In detail, both stem parts and medium were homogenized with an Ultraturrax® mixer coupled to a disposing tool (Ika® Werke T8.01 S8N-5G, Staufen, Germany) until a green viscous mass was obtained. An extraction with 20 mL acetonitrile/water (84/16, v/v) and a hexane defatting (10 mL) was performed using the Agitator decanter overheadshaker (Agitelec, J. Toulemonde & Cie, Paris, France) for 60 minutes for the whole procedure. The extraction solvent and the hexane were added simultaneously. The aim of defatting with hexane was executed mainly to remove the chlorophyll. Acetonitrile (Analar Normapur) and n-hexane (Hipersolv Chromanorm) were obtained from VWR International (Zaventem, Belgium).

Subsequently, stem parts and the viscous mass medium were centrifuged at 3,000 g for 15 minutes, afterwards, the supernatant (hexane layer) was removed, while the aqueous layer was filtered through an Ederol-filter paper (12.5 cm, quality 15, VWR, Belgium). The filtrates were evaporated to dryness under a gentle N_2-stream at 40 °C using an evaporator module (Grant Instruments Ltd, Cambridge, United Kingdom), and were redissolved in 100 µL injection solvent, consisting of mobile phase A and mobile phase B in a 50/50-ratio. Mobile phase A consisted of water/methanol (95/5, v/v (A)) and B of methanol/water (95/5, v/v (B)), both buffered with 10 mM ammonium acetate and adjusted to pH 3 with glacial acetic acid. The pH of the aqueous component was adjusted before mixing with the organic component. Methanol (LC-MS grade) was purchased from BioSolve BV (Valkenswaard, The Netherlands), while ammonium acetate was obtained from VWR International (Zaventem, Belgium). Acetic acid (glacial, 100%) was supplied by Merck (Darmstadt, Germany). Finally, the redissolved sample was vigorously vortexed for 3 minutes, collected in an Ultrafree-MC centrifugal device (0.22 µm; Millipore, Bedford, MA, USA) and centrifuged for 10 minutes at 10,000 g. Samples were stored in vials in the dark at 4 °C until analysis. Matrix blanks, to which no mycotoxin was added were prepared similarly.

Liquid chromatography-tandem mass spectrometry (LC-MS/MS) conditions

The method used for the analysis of DON-3G has been detailed in De Boevre *et al.* (2012) (De Boevre *et al.*, 2012b). LC-MS/MS analysis was performed using a Waters Acquity UPLC system coupled to a Quattro Premier XE mass spectrometer (Waters, Milford, MA, USA). MassLynx™ version 4.1. and QuanLynx® version 4.1. software (Micromass, Manchester, UK) were used for data acquisition and processing.

Liquid chromatography-high resolution mass spectrometry (LC-HRMS) conditions

Concerning unknown T-2 conjugates, LC-HRMS was applied using the LC-Orbitrap MS Exactive™ (ThermoFisher Scientific, San José, USA) according to the method described in De Boevre *et al.* (2012) (De Boevre *et al.*, 2012b). In detail, chromatographic separation was performed applying a ZORBAX Eclipse XDB C18-column (3.5 µm, 100 mm x 4.6 mm) (Agilent Technologies, Diegem, Belgium). The column was kept at room temperature (21.5 °C; range [21 °C-22.4 °C]), while the autosampler was set at 10 °C. A mobile phase consisting of water/methanol (95/5, v/v (A)) and methanol/water (95/5, v/v (B)), both buffered with 10 mM ammonium acetate and adjusted to pH 3 with glacial acetic acid was used at a flow rate of 0.2 mL min^{-1}. Mobile phases were prepared by firstly adjusting the aqueous ammonium acetate-solution to the proper pH. After the addition of the organic solvents, the pH was controlled and the solutions were re-adjusted. The gradient elution

program started at 50% mobile phase A for 2 min with a linear increase to 100% mobile phase B in 8 min. A flow of 100% mobile phase B was initiated at 10 min for 5 min. Before analysis, the mobile phases were filtered through a 0.22 µm PVDF-filter. The duration of each HPLC run was 20 minutes, including reequilibration. The HPLC system was composed of an Accela pump and a Thermo PAL auto injector (ThermoFisher Scientific, San José, USA).
The MS parameters included the following settings: ESI source block temperature 150 °C; desolvation temperature 300 °C and capillary voltage 4 kV. Other MS parameters were further optimized during analysis. Other parameters like capillary temperature (275 °C), capillary voltage (67.5 V), tube lens voltage (120 V), skimmer voltage (18 V) and heater temperature (200 °C) were optimized. Indeed, after setting these parameters an increase of 62% of the signal was observed. The electrospray positive (ESI^+) operation mode was appointed. Scanning in the mass range of *m/z* 80 to 1000 at a resolving power of 100,000 full width at half maximum (FWHM) was set. A full scan with collision induced dissociation (CID) was performed in different chromatographic runs. The mass spectrometer was capable of generating fragmentation information in a non-selective manner using a collision cell without precursor ion selection. The Orbitrap technology provided that ions passed through the C-trap into a multipole collision cell where they were fragmented. Thereafter, ions were transferred into the C-trap from where they were injected into the Orbitrap for detection. For data acquisition Xcalibur® software (Thermo Scientific, San José, USA) was applied.

3. Results and discussion

The newly developed method is based on the assumption that detached leaves are able to metabolize mycotoxins. Before testing the method on T-2, the assumption was successfully validated on DON. Calibration standards for DON-3G were on hand, therefore LC-MS/MS could be used as a confirmatory method on detached leaves fed with the precursor DON. HRMS was then further on applied for the detection of unknown conjugates of T-2. The application of the detached leaf *in vitro* model and subsequent biosynthesis of DON-3G and masked forms of T-2 is detailed in the following section.

Biosynthesis of DON-3G

DON-3G was determined according to the LC-MS/MS method described (De Boevre *et al.*, 2012b). (**Figure 2**). The Chinese cultivar Sumai3 was utilized for the glucosylation tests of DON with wheat. This cultivar has previously been described to have a significant resistance against DON and to contain a QTL associated with DON glucosylation, designated as the *Fhb1* QTL (Lemmens *et al.*, 2005). Interestingly, no DON-3G could be detected in the liquid medium or plant extract after application of 1,000 µg kg^{-1} DON, while detection was only possible after feeding up to 10,000 µg kg^{-1} DON.
This role for DON as a proportional trigger for glucosylation is well in accordance with Audenaert *et al.* (2013) (Audenaert *et al.*, 2013). These authors investigated glucosylation capacities in both an artificial inoculation experiment and a field trial under natural circumstances. While DON levels were obviously very different between the two set-ups, the relative glucosylation capacities (expressed as share of DON-3G to the sum of DON and DON-3G) were very similar. These authors found that in the grains of naturally contaminated wheat, levels of DON approximately 1,000 µg kg^{-1} were steadily associated with DON-3G levels of 150 µg kg^{-1} to 300 µg kg^{-1}. Detached leaves are clearly less effective at glucosylating DON than wheat ears.
However, addition of 10,000 µg kg^{-1} DON to the detached wheat leaf assay did lead to detectable DON-3G levels. Unfortunately, biological variation over three repetitions was quite large. Several factors may have influenced this variation, such as difference in weight of the leaf fraction between the repetitions, and discrepancies in maturity of the various leaf

parts. Detection of DON-3G levels of 100 µg kg^{-1}, 200 µg kg^{-1} and 1,000 µg kg^{-1} was registered.

Biosynthesis of masked forms of T-2

The obtained results for DON indicate that the model system using detached leaves of wheat is able to produce the known metabolite DON-3G. It was the authors' goal to subject the highly toxic trichothecene T-2 to the validated *in vitro* system.

Prior to the analysis of T-2 spiked detached leaves, the HRMS equipment was tuned for optimal detection of T-2 conjugates, using two working solutions containing T-2 (10 ng μL^{-1} and 100 ng μL^{-1}). Radio frequency and voltage parameters were adjusted to gain a maximum mass transfer of the *C*-trap to the Orbitrap. For an optimal detection and stable electrospray, the T-2 standard was tuned. The authors assumed that tuning of the T-2 standard would give optimal results for the detection of T-2 metabolites. A spectrum was obtained showing a peak at mass to charge ratio (*m/z*) of 484.25. Possible according molecular masses for 484.25 were determined (**Table 1**). The exact mass of $C_{24}H_{38}O_9N$ was set at taking the $[M+NH_4]^+$ adduct into account. Ideal parameters for T-2 were necessary to be found because conjugates of T-2 were searched.

Additional post-data acquisition mathematical tools using the Xcalibur® software were applied to perform molecular identification. According to all possible molecular weights (MW) of conjugation products including positive adducts (H^+, NH_4^+, Na^+ and K^+) of both T-2 and HT-2, a database was established to extrapolate data obtained from the inoculated stem parts (100 µg kg^{-1} and 1,000 µg kg^{-1}).

Full scans from *m/z* 80 to *m/z* 1,000 were recorded. The obtained chromatograms were investigated and peak values were determined. Interference peaks were recorded at *m/z* 157 and *m/z* 179, which could be attributed to the DMSO used as a solvent for T-2. To obtain higher efficiency, the full scan was recorded from *m/z* 200 to *m/z* 1,000. Besides peaks relevant to T-2 and HT-2, three peaks were attributable to the mono-glucoside derivatives of T-2 and HT-2, and the triglucoside of T-2 (T-2-(3)G_3). The measured MW 604.29593 was attributed to the molecular formula of $C_{28}H_{46}O_{13}N$, corresponding to [HT-2-glucoside + NH_4]$^+$ with a mass accuracy of -0.52002 ppm (intensity of 5.77 x E^7). The mass value of 646.30665 suggested to be [T-2-glucoside + NH_4]$^+$ with molecular formula of $C_{30}H_{48}O_{14}N$ with a mass accuracy of -0.50991 ppm (intensity of 2.82 x E^7). NH_4-adducts appear to be the most prevalent in the system. The earlier elution of T-2-(3)G_3 (8.99 min) compared with those of T-2-(3)G (9.87 min) and T-2 (10.85 min) suggests that T-2-(3)G_3 is more hydrophilic than T-2-(3)G and T-2. The mass value of T-2-(3)G_3 (MW 971.45321, $C_{42}H_{68}O_{24}N$) was detected at a reasonable deviation (-1.06 ppm). Exact mass values of the ions were pointed out and in accordance with the European Commission guideline, mass deviation <5 ppm from the theoretical value was used as the criterion for compound identification (ISO/IEC 17025, 2011).

Previous reports described the presence of T-2-diglucoside (T-2-(3)G_2) and HT-2-diglucoside (HT-2-(3)G_2), however ions with MW 808 and 766 could not be extracted. Full scans were scrutinized with the calculated masses of HT-2-(3)G and T-2-(3)G, and clear peaks at 9.00 min and 9.87 min were detected (**Figure 3** and **4**, respectively). The analysis was repeated twice with the recording of full scans from *m/z* 500 to *m/z* 700 and *m/z* 800 to *m/z* 1,000.

Assuming that similar fragmentation occurs for T-2, T-2-(3)G and T-2-(3)G_3, a full scan chromatogram for all observed forms was acquired using fragmentation by the collision cell (collision energy, 10 eV). Due to absent reference standards for the T-2-glucosides, the authors have assumed that the glucosides have similar MS responses to those for T-2 and HT-2. The hypothesis of the structures was confirmed by the presence of main fragment characteristics, as detected in the spectrum of the precursor ion. Neutral losses in

trichothecenes are mostly observed (Berger *et al.*, 1999). The loss of isovaleric acid, acetic acid, formaldehyde and the glucose-fragment was proven by measuring mass values of 544.1562, 514.5123, 484.4874 (T-2-(3)G); 502.6351, 442.1478 and 412.3265 (HT-2-(3)G), respectively. For T-2-(3)G_3 similar fragments were formed with the inclusion of the neutral loss of the glucose fragment, 646.2368. In previous studies, it was assumed that glucosylation of HT-2 mainly occurs at C_3 rather than at C_4 because T-2 consists of only one OH at C_3 and was therefore concurrently glucosylated. **Figure 5** illustrates the spectrum of [T-2-(3)G_3 + NH_4]$^+$. Peaks for 940.4, 950.4 and 955.4 in the spectrum were not characterized. **Figure 6** illustrates the structure of T-2 (left) and the assumed structure for T-2-(3)G_3 (right). No NMR measurements of the T-2 and HT-2 conjugates were recorded as the available quantity of these forms were too low.

The analysis of detached leaves spiked with T-2 toxin allowed for the first time detection of a triglucosylated form of T-2 toxin. Previous findings (Lattanzio *et al.*, 2012; Veprikova *et al.*, 2012; Nakagawa *et al.*, 2011) proved the widespread presence of trichothecene glucosides in cereal grains. The results of this study underscore the importance of the conjugation mechanism *in planta*.

Quantification of the T-2 masked forms was not possible due to the lack of reference standards, however, based on the observed peak areas the formation was estimated to be less than 10% of the parent toxin. According to these estimations, glucosylation rates in the detached leaf model showed similar results of previous reports of other trichothecene glucosides in wheat, while it must be kept in mind that the detached leaves are only moved to glucosylation at a higher dosage of toxin addition than wheat ears.

In conclusion, DON-3G was confirmed to be present in the newly-developed detached leaf *in vitro* model, designating the system as a useful tool to synthesize glucosylated conjugates, especially for the trichothecene-family. The existence of mono-glycosylated T-2 and HT-2 and a triglucoside of T-2 was endorsed by means of LC-Orbitrap MS. The newly described triglucoside of T-2 could also be found in this set-up, nicely illustrating the usefulness of the detached leaf model. The insertion of other parent mycotoxins to the set-up will in the future allow for identification of even more conjugates. This method provides financial and time-efficient advantage, as the untargeted screening can be limited to a few iterations of the developed *in vitro* system, while the information on new metabolites gleaned from this HRMS analysis, can then be used in toxicological studies and targeted routine screening of field samples.

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FIGURES

Figure 1. Illustration of the set-up of the detached leaf *in vitro* model in maize. The picture shows the stems of the maize plant with the medium (left) and the viscous mass after mixing with the Ultraturrax® (right)

Figure 2. Flow of the investigation on DON-3-glucoside (DON-3G) with the total ion chromatogram of the reference standard, and MRM-channels of DON-3G in the detached leaf *in vitro* model.

Figure 3. Flow of the investigation on HT-2-(3)-glucoside (HT-2-(3)G) with the total ion chromatogram in the detached leaf *in vitro* model. The total ion chromatogram was scrutinized and a first extracted chromatogram was elaborated at 9.00 minutes. A second, more detailed chromatogram was pointed out of the first chromatogram to perfectly obtain a clear peak. After software-analysis, a spectrum was obtained, indicating the mass of HT-2-(3)G.

Figure 4. Flow of the investigation on T-2-(3)-glucoside (T-2-(3)G) with the total ion chromatogram in the detached leaf *in vitro* model. The total ion chromatogram was scrutinized and a first extracted chromatogram was elaborated at 9.87 minutes. A second, more detailed chromatogram was pointed out of the first chromatogram to perfectly obtain a clear peak. After software-analysis, a spectrum was obtained, indicating the mass of T-2-(3)G.

Figure 5. Full scan spectrum of the ammonium adduct of T-2-(3)-triglucoside [T-2-(3)G_3 + NH_4]$^+$ in the detached leaf *in vitro* model.

Figure 6. Structure of T-2 toxin (left) and the assumed structure of T-2-(3)-triglucoside (right)

TABLES

Table 1. Elemental composition search on mass 484.25 (*m/z* 479.25 - 489.25). The bold value indicates the chosen composition of $C_{24}H_{38}O_9N$, namely T-2-toxin