Chapter 2.

Role of fungal endophytes on pyrrolizidine alkaloids metabolism in *Jacobaea* plants

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ABSTRACT

Pyrrolizidine alkaloids (PAs) are known as plant defence compounds with highly diverse structures. In *Jacobaea* plants, PAs have been observed to be diverse in both concentration and composition. This diversity may be influenced by biotic and abiotic factors. Plant fungal endophytes are part of the biotic environment. In this study, we eliminated fungal endophytes by treating the plants of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* with three fungicides: Folicur, Pronto Plus and Switch. As a result, no detection of fungal endophytes was observed in the Pronto Plus and Switch plants. A fungal endophyte was detected in the Folicur plants. Based on the b-tubulin gene and the Internal Transcribed Spacer (ITS) region of the rDNA, the detected fungus had a close homology with mycorrhizal fungi belonging to the Glomeromycota. The PA content was measured using LC-MS/MS. The Folicur-treated plants showed a lower total PA amount compared to the control whereas the other treatments showed no differences. This suggests that *de-novo* PA synthesis in *Jacobaea* plants is not dependent on endophytes. However, the endophyte present in the Folicur treatment lowered the total amount of PAs produced and changed their composition, leading to relatively low amounts of senecionine- and jacobine-like PAs in contrast to erucifoline- and otosenine-like PAs. In this way endophytes may increase the variation of PAs in *Jacobaea* plants possibly leading to consequences in plant defence against generalist and specialist pathogens and herbivores.

Key Words: *Senecio*, *Jacobaea vulgaris*, *Jacobaea aquatica*, pyrrolizidine alkaloids, fungal endophyte, fungicides.

INTRODUCTION

Plants produce a diversity of secondary metabolites to interact with their dynamic, often hostile environment. These metabolites are distinct from the components of primary metabolism and are not essential for the basic metabolic processes of the plant(Dixon, 2001;Hartmann, 2007). Secondary metabolites may function as a defence against herbivores and pathogens. Pyrrolizidine alkaloids (PAs) belong to one of the secondary metabolite groupsinvolved in the plant's constitutive chemical defence (Ober and Kaltenegger, 2009; Macel et al., 2005).This group of alkaloid is known for its high diversity in occurrence and chemical structure. Pyrrolizidine alkaloids are present in distantly related families such as Asteraceae, Boraginaceae, Fabaceae and Orchidaceae (Hartmann, 1999). Many studies have been conducted to understand the role and meaning of this diversity for plant survival. Accumulated evidences emphasise the importance of both biotic and abiotic factors in PAs diversity.

Pyrrolizidine alkaloids are esters of a necine base with one or more necic acids (Hartmann, 1999). PAs occur in two interchangeable forms: the free base (tertiary amine) and the *N*-oxide form (Hartmann and Dierich, 1998). The PAs in *Jacobaea* plants have been studied intensively and have been reported to be very diverse in both concentration and composition (Pelser et al., 2005; Witte et al., 1992; Macel et al., 2004). At least 37 PAs have been reported from 24 species of the genus *Jacobaea* (Cheng et al., 2011; Pelser et al., 2005). Based on the biosynthesis route, these are dividedinto four main groups : senecionine-, jacobine-, erucifoline- and otosenine-like PAs (Pelser et al., 2005). The PA composition of species such as *Senecio vernalis*, *Senecio vulgaris*, *Senecio inaequidens*, *Jacobaea erucifolia* and *Jacobaea vulgaris* are different (Hartmann and Dierich, 1998). Even within a species, the variation of PA composition like in *J. vulgaris* is high. This intra-specific variation is described by four different chemotypes i.e. the jacobine, erucifoline, senecionine or mixed types (Macel et al., 2004; Witte et al., 1992). PA concentrations differ between organs. For example in *S. vulgaris*, the concentration of PAs in inflorescences is the highest, followed by the leaves, roots and stem (Hartmann and Zimmer, 1986). Composition of PAs differs between organs as well. Generally, the shoots contain higher proportions of jacobine- and erucifoline-like PAs and lower proportions of senecionine- and otosenine-like PAs compared to the roots (Joosten et al., 2009). The PA composition is affected by hybridization (Cheng et al., 2011), plant development stage (Schaffner et al., 2003), and by abiotic factors such as nutrients (Hol et al., 2003), soil types (Joosten et al., 2009), as well as environmental conditions (Hol et al., 2003; Macel and Klinkhamer, 2009, Kirk et al., 2010; Joosten et al., 2009). Furthermore the biotic environment including herbivorous insects (Macel et al., 2005;Leiss et al., 2009), plant fungi(Hol and van Veen, 2002; Hol, 2003) and soil born microorganisms (Joosten et al., 2009; Hol, 2003) can have an effect on PA composition.

Plant endophytes form part of the biotic environment. Plant endophytes refer to bacterial or fungal microorganisms living within plants without causing any visible symptoms or pathogenic effects to the host (Gunatilaka, 2006). Fungi represent the largest group among endophytic microorganisms (Carroll, 1988). Endophytic fungi are ubiquitous and can be extremely diverse in host plants. Almost every organ from every plant species examined harbours at least one species of endophytic fungus (Petrini, 1991). Involvement of endophytic fungi in the biosynthesis of

alkaloids has been observed in several plants. The endophytic fungus *Fusarium solani* produces a quinoline alkaloid, camptothecin, in C*amptotheca acuminata* (Happy Tree plants) (Kusari et al., 2011). *Ipomoea asarifolia* (Ginger Leaf Morning Glory) infected with the endophytic fungus Clavicipitaceous synthesises ergoline alkaloids (Ahimsa-Müller et al., 2007). Another example is loline, a fungal alkaloid, produced in Cool Season grasses (Poaceae; subfamily Pooideae) which are infected by an endophytic fungus of the genus *Epichloë* (Clavicipitaceae) and its asexual derivative, the *Neotyphodium* species (Schardl et al., 2004). In general, the symbiotic relationship between fungi and host plants is a mutualistic one since the hosts provide the protection and food for the fungi, whereas in turn the fungi support the host in production of bioactive secondary metabolites (Gunatilaka, 2006). However, more recent evidence suggests that an interaction between host and endophytic fungi does not have to be mutualistic (Jaber and Vidal, 2010; Clay, 1996). It may also be neutral (Saikkonen et al., 1999) or antagonistic (Vicari et al., 2002). Most likely, the type of symbiotic relationship depends on the type of endophytic fungi, plant genotype and environmental conditions (Faeth and Fagan, 2002).
Although plants belonging to the *Jacobaea* genus have been studied intensively, the effect of endophytic fungi on PAs synthesis inthis plant genus is still unknown. Fungal endophytes can be eliminated by treating plants with systemic fungicides. Currently, there are many kinds of synthetic fungicides commercially available with different mode of actions targeting fungal respiration, osmoregulation, methionine or sterol biosynthesis (Leroux et al., 2002).

In this study, we aimed to determine a possible role of endophytic fungi in the biosynthesis of PAs in *Jacobaea* plants. We, therefore, applied different fungicide treatments and studied their effect on PA amount and composition. In addition, we investigated a possible non-specific effect of the fungicide treatments on the plants using a metabolomic approach.

MATERIALS AND METHODS

Set Up of Experiments. F2 hybrid plants of *Jacobaea vulgaris* and *Jacobaea aquatica* were chosen as a study system. Detailed information about the origin of the parent plants and how the hybrids developed had been reported by Cheng et al. (2011). In contrast to the parent plants *J. vulgaris* and *J. aquatica* which accumulate jacobine- and senecionine-like Pas, respectively, F2 hybrids contain all four PA types in comparable proportions. The plants were treated with three different systemic fungicides which have been successfully used to eliminate endophytic fungi in *Ipomoea asarifolia* (Convolvulaceae) (Kucht et al., 2004). The fungicides used were Folicur, Pronto Plus and Switch, exhibiting different ranges and modes of action. Folicur contains 251 g/l tebuconazole as an active ingredient. This active compound claims a broad-spectrum of activity, although it was specifically designed for control of powdery mildew (Ascomycota) and rust fungi (Basidiomycota). Pronto Plus contains 133 g/l tebuconazole and 250 g/l spiroxamine. The addition of spiroxamine suggests that Pronto Plus has a broader spectrum of activity than Folicur. Pronto Plus and Folicur are fungicides that inhibit sterol biosynthesis. Switch contains two active ingredients, 375 g/l cyprodinol and 250 g/l fludioxonil. These anilinopyrimidine fungicides are effective against various Ascomycetes and Adelomycetes. The activity is based on inhibition of methionine biosynthesis and secretion of hydrolytic enzymes (Leroux et al., 2002).

Fungicide Treatment. The F2 hybrid plants were obtained from the tissue culture collection of our department and were transplanted into pots filled with 1:1 sand and soil. Plants were kept in a climateroom (humidity 70%; light 16 h at 20°C; dark 8 h at 20°C). The fungicide concentrations applied were 0.15% for Folicur and Pronto Plus, and 0.1% for Switch. Plants were sprayed with fungicides when six-weeks old. Before spraying five plants were harvested. We refer to these plants as starting materials. For each fungicide treatment, five plants were used. In addition to the treatments, five plants were sprayed with water and these served as control. The plants sprayed with Folicur are referred to as Folicur plants, while plants sprayed with Pronto Plus or Switch are referred to as Pronto Plus and Switch plants, respectively. The fungicides were applied four times with intervals of two weeks in between. One day after the last spraying all plants treated with fungicides as well as the control plants were harvested.

Plant Harvesting. Whole plants including shoots and roots of twelve-weekold plants were removed from the pots, washed, and then frozen in liquid nitrogen. The frozen plants were crushed with mortar and pestle and freeze-dried. The dried samples were weighed and subsequently the samples were kept at -80 ºC until further work-up including endophytes detection, PA analysis and NMR based metabolomics.

*EndophyteDetection***.** To detect the presence of endophytic fungi in the plants, we amplified genes specific for fungi using primers of the β-tubulin gene and the internal transcribed spacer (ITS) region of the rDNA gene cluster. Total DNA was extracted from dry plant material using the Dneasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands). The amplification of the partial β-tubulin gene was carried out using Bt3- LM and Bt10-LM primers (Myllys et al., 2001). Amplification of the ITS region of the rDNA gene cluster was performed with the primers ITS5 and ITS4 (White et al., 1990). Total DNA of *Fusarium oxysporum* (IBL collection) was used as a positive fungal control. Amplification was performed in 20 µl aliquots consisting of 2 µl 10x PCR buffer, 0.5 µl 25 mm $MgCl_2$, 0.5 µl dinucleotide triphosphates (dNTPs) containing 10 µM of each base, 1 µl of each primer 10 µM, 0.2 µl 5 U/µl *Taq* polymerase (QIAGEN, Venlo, The Netherlands) and 1 µl of stock DNA. The PCR conditions were: 3 min at 95 °C; denaturation 30 s at 95 °C, annealing 1 min at 55 ° C (for the ITS region gene) or 1 min at 59 ° C (for the partial β-tubulin gene), extension 1 min at 72 °C (40 cycles), and 15 min at 72°C. The PCR products were separated in a 1.5% agarose gel for evaluation of the product sizes. We then sequenced the β-tubulin and ITS PCR products derived from the control, Folicur plants and the positive fungal control (MACROGEN, Amsterdam, The Netherlands) to identify the genes amplified. We only sequenced the Folicur plants since they were the only treatment containing two ITS PCR products similar in size to that of the control plants and positive fungal control. Alignment assembly and phylogenetic analyses were performed with Geneious 5.5.6 (Biomatters development teams) software. The resulting alignments of the β-tubulin -(701 nucleotides) and the ITS gene (712 nucleotides and 550 nucleotides) were used to retrieve similar sequences from the databases in BLAST searches (Altschul et al., 1990) at the NCBI server http://www.ncbi.nlm.nih.gov/ blast/Blast.cgi. Several sequences, with the closest similarity to our sequences, deriving from different species, were chosen to construct a phylogenetic tree. The fungi *Yarrowia lipolytica* and *Boopis anthemoides* belonging to the *Saccharomycetes* were chosen as additional entries from less closely related fungi to highlight phylogenetic distances. Phylogenetic trees were based on the Jukes-Cantor distance. The bootstraps of the phylogenetic tree were 50% with 100 replicates.

Pyrrolizidine Alkaloid Content. We extracted PAs using aqueous acidic conditions and subsequently measured PAs using liquid chromatography–tandem mass spectrometry (LC-MS/MS). As extraction solvent 2% formic acid was used. Heliotrine at a concentration of 1 µg/ml was added as internal standard. Approximately 10 mg of dried plant powder was extracted by shaking for 1 h with 1 ml of extraction solvent. Solid substances were removed by centrifugation at 9000 rpm for 10 min, followed by filtering the supernatant through a 0.2-µm nylon membrane (Acrodisc 13-mm syringe filter, Pall Life Sciences, Ann Arbor, MI, USA). An aliquot of 25 µl of the filtrate was diluted with 975 ml of water and injected into the LC-MS⁄MS system. The system consisted of a Waters Acquity ultra performance liquid chromatographic (UPLC) unit coupled to a Waters Quattro Premier XE tandem mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation conditions followed the ones reported by Cheng et al. (2011b). Seventeen individual PA standards (see Joosten et al., 2011, for the source of the standards) were available for this study. For compounds for which no reference standard was available, a semi-quantitative, i.e. indicative value was obtained by comparison with the most closely related isomer. Identification of PAs was based on their retention time, molecular mass and fragmentation pattern and comparison with PA standards and ⁄ or literature data. Data processing was conducted using Masslynx 4.1 software (Waters, Milford, MA, USA). The total PA concentration was defined as the sum of all detected individual PAs. To obtain the amount of PAs per plant, total PA concentration was multiplied with the corresponding plant biomass (dry weight). Except for senecionine *N*-oxide, which is regarded the precursor of all other PAs (Hartmann and Dierich, 1998),we classified the PAs into four groups according to their structural characteristics and biosynthetic pathways (Pelser et al., 2005): senecionine-, jacobine-, erucifoline- and otosenine-like PAs. Each PA group included both the tertiary amine and *N*-oxide forms. The amount of each group of PAs was determined by the changes in the control and treated plants relative to the starting material, thus representing PA production during the period of treatment. The relative concentration of each PA type was calculated as: (absolute concentration of PA type ⁄ total PA concentration) × 100.

NMR Metabolomics. To detect effects on fungicide treatment on the plant metabolome in general, we applied NMR metabolomics. For this 50 mg of dried and powdered material of each plant was extracted using a mixture of KH_2PO_4 buffer (pH 6.0) in D_2O and methanol-d_4 in a ratio of 1:1. The extraction followed the standard NMR based metabolomics procedure for plants (Kim et al., 2010). NMR spectra were recorded at 25°C on a 600 MHz Bruker DMX-600 spectrometer (Bruker, Karlsruhe,Germany) operating at a proton NMR frequency of 600.13 MHz. The NMR conditions followed the ones previously described for *Senecio* plants by Leiss et al. (2009). For the metabolomic analysis, signal intensities of ^{1}H NMR spectra were scaled to total intensity and reduced to integrated regions of equal width (0.04 ppm) corresponding to δ 0.4-10.0. The regions of δ 4.75-4.9 and δ 3.28-3.34 were excluded from the analysis because of the residual signal of D_2O and CD_3OD, respectively. Bucketing was performed by AMIX software (Bruker, Karlsruhe,Germany). The quantification of the identified metabolites was performed by scaling the intensity of the ^{1}H NMR to the signals of TMSP at 0.00 ppm. Data obtained were subjected to multivariate analysis in order to determine differences in metabolomes between samples.

Data Analysis. The amount of total PA, senecionine *N*-oxide and the different PA-types were analysed using ANOVA with fungicide treatments as a fixed factor and Duncan's multiple

comparisons as a post-hoc test. This statistical analysis was performed using SPSS 19.0. Partial Least Square-Discriminant Analysis (PLS-DA), a supervised multivariate data analysis, was used to cluster the samples based on the relative PA concentrations. This multivariate analysis was performed using SIMCA-P software (version 12.0, Umetrics, Umeå, Sweden). Scaling for PLS-DA was based on the Pareto method. The models were validated by permutation tests through 20 applications and CV-ANOVA, which are the default validation tools in the software package (SIMCA-P). The loading plots were used to identify which ^{1}H NMR signals were important factors for the clustering. The compounds responsible for the clustering were quantified and analysed using ANOVA.

RESULTS

Plant Dry Weight. At harvest, all plants, i.e. the six-week old starting material and the twelve-week old control and fungicide treatments were in the vegetative rosette stage. Dry mass of the starting group, harvested before the treatment, was significantly lower compared to the twelve-week old plants (ANOVA, $F=8.797$, $df= 4$, $P<0.001$). No significant differences in dry weight were observed among the control and fungicide treatments (data not shown).

Endophyte Detection. Endophytic fungi could only be detected in the Folicur plants as indicated by the amplification of the 750 kb β-tubulin gene (Fig.1a). This PCR band was consistently observed in all replicates of the Folicur plants. Amplification of the ITS region of the rDNA gene cluster resulted in a 800 kb DNA fragment in all of the samples. Aside from that, a 600 kb size ITS DNA fragment was observed in the Folicur plants. The DNA fragment had a similar size as the ITS DNA fragment of *F. oxysporum*, which had been used as positive fungal control (Fig. 1b). This indicated that the Folicur plants contained an endophytic fungus in higher concentrations compared to the other treatments. No DNA fragments with similar sizes to these fungal *F.oxysporum* fragment were observed in neither the starting material nor the control, Pronto Plus or Switch plants.

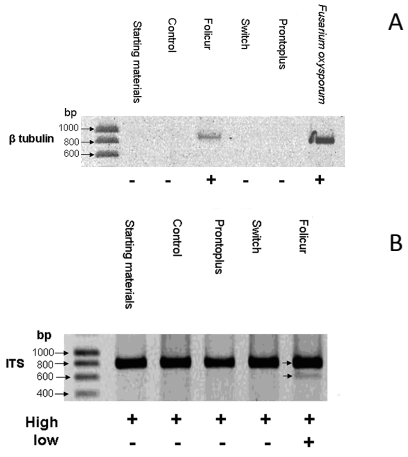

Fig 1. Qualitative analysis of the β tubulin gene (A) and ITS region of the rDNA gene cluster (B) in F2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris* after fungicide treatment. + indicates presence and – indicates absence of the corresponding genes. The visible PCR products indicate the concentration of related DNA > 0.28 ng/ml.

Validation of the gene primer showed that the β-tubulin sequence obtained from the positive control *F.oxysporum* showed a high similarity (99% identity) with the β-tubulin sequence of the corresponding accession (AB587041.1) in the NCBI database. The 600 kb partial β-tubulin sequence observed in the Folicur plants was grouped in the same clade as *Glomusclarum,* belonging to the Glomeromycota division, and *Corynespora smithii* belonging to the subdivision of the Pezizomycotina being part of the Ascomycota division (Fig. 2a). The 800 kb sequence of the ITS 4-5 region was observed in the control and treatment samples including Folicur. This sequence was grouped in the same clade as the ITS 4-5 regions of several *Jacobaea* and *Senecio* plants including the parents of the F2 hybrids used in this study: *J. vulgaris* (FJ556936.1) and *J. aquatica* (FJ556950.1) (Fig. 2b). The smaller ITS DNA fragment at 600 kb was grouped in the Glomeromycota clade but also occurred in the Phoma sp. belonging to the Ascomycota. (Fig. 2c). Thus both sequences, the ITS and the β-tubulin sequence indicated that the fungus detected in the Folicur plants, were closely related to the *Glomus* genus within the Glomeromycota division.

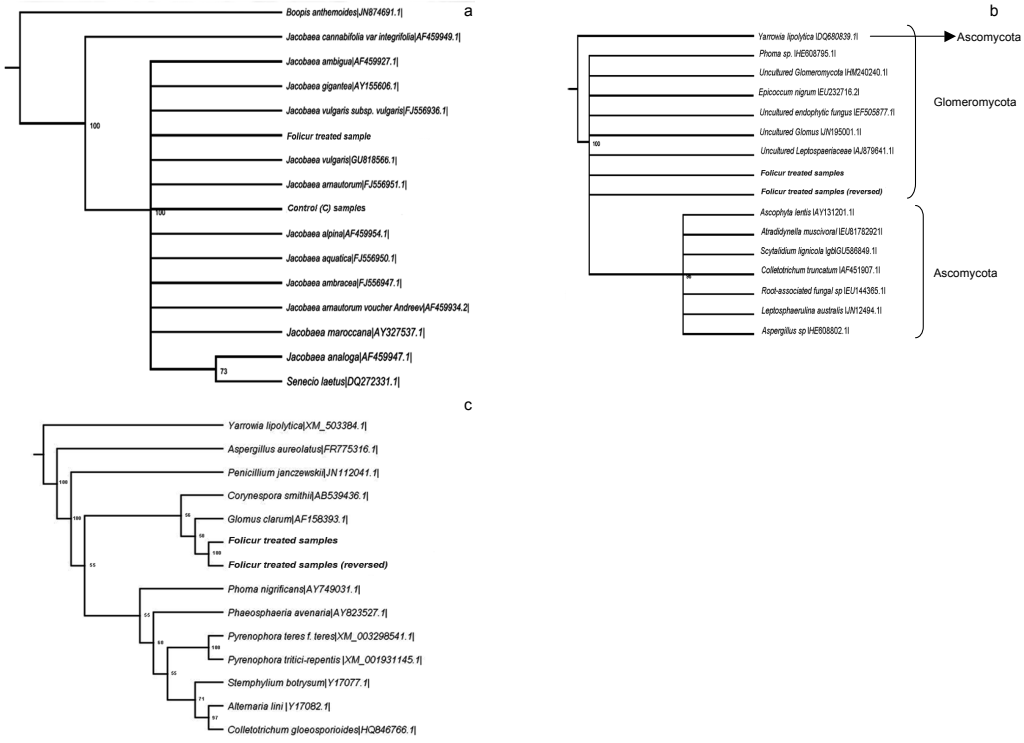

Fig 2. Phylogenies of the ITS sequences at 800 bp (A), and 600 bp (B), as well as of the β tubulin sequences at750 bp (C). Sequences were compared with the highest similarity sequences available in the NCBI database obtained by BLAST. Phylogenetic trees presented are neighbor joining trees based on the Jukes-Cantor distance. The bootstraps were 50% with 100 replicates. *Yarowia lipolytica* and *Boopis anthemoides* were chosen as additional entries from less closely related fungi to highlight phylogenetic distances.

Pyrrolizidine Alkaloid Content. The twelve-week old control group showed a significantly higher total PA concentration compared to the six-week old starting material. The Folicur-treated plants contained lower total PA content compared to the control plants, while the Pronto Plus and Switch plants contained similar amounts of PAs as the control (ANOVA, F=8.741, df= 4, P<0.0001) (Fig. 3a).

The amount of the different PAs, including senecionine *N*-oxide, and the groups of senecionine-, jacobine-, erucifoline- and otosenine-like PAs, showed significant differences between treatments (Fig. 3b). The highest amount of senecionine *N*-oxide was observed in the Switch plants, an intermediate amount in the Pronto Plus and the lowest amount in the Folicur plants and the control (ANOVA, F=9.91, df= 4, P<0.0001). The amount of senecionine-like PAs (without senecionine *N*-oxide) was significantly lower in the Folicur plants compared to all other treatments and the control (ANOVA, F=6.83, df= 4, P=0.001). The same pattern was observed for jacobine-like PAs showing significantly lower amounts in the Folicur plants (ANOVA, F=4.26, df =4, P=0.012). The amount of erucifoline-like PAs in the Folicur plants was significantly lower compared to the other two fungicides treatments but not compared to the control (ANOVA, F=11.72, df= 4, P<0.0001). Otosenine-like PAs occurred only in relatively low amounts in all plant groups, significantly lower in Folicur plants compared to Switch, but did not differ significantly from the Pronto Plus plants or the control respectively (ANOVA, F=4.62, df= 4, P=0.008).

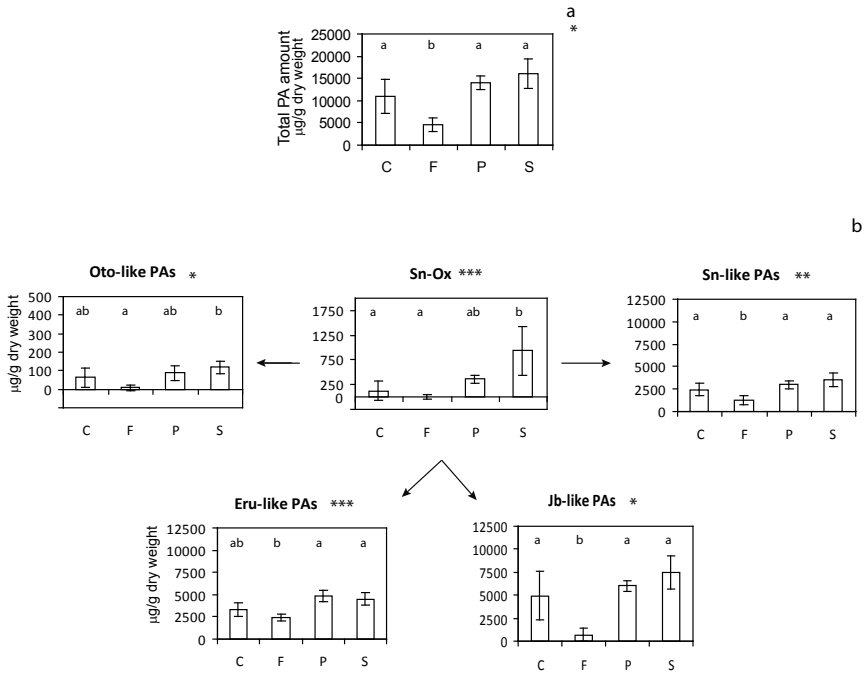

Fig 3. Total PA concentration of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* (A). Senecionine *N*-oxide (Sn-ox) and four types of PAs including, senecionine (Sn)-, jacobine (Jb)-, erucifoline (Eru)- and otosenine (Oto)-like PAs composed the total PA amount in each treatment (B). The arrows show that the synthesis of the PAs types is derived from senecionine *N*-oxide. Data present the mean of 5 replicates and the standard error. Treatments were coded as: St=Starting material; C=Control; F= Folicur treatment; P= Pronto Plus treatment; S=Switch treatment. Different letters indicate means that are significantly different at * *P*< 0.05, ** P< 0.01, *** *P* < 0.001.

Partial least square-discriminant analysis (PLS-DA) showed a clear separation between the control and the Folicur plants (Fig. 4). PA-types explained 60% of the total variation between treatments. The control was located in the positive quadrant of PC1 while Folicur plants were located in the negative quadrant. The Pronto Plus and Switch treatments as well as the starting material were located in the middle of the score plot (Fig. 4). PC3 specifically separated the control and Folicur plants from the starting material.The loading plot of the PLS-DA was used to investigate which types of PAs differentiated Folicur from the control. Erucifoline-like PAs were located in the negative quadrant of PC1 coinciding with the Folicur plants. In contrast, the jacobine- and senecionine-like PAs were located in the positive quadrant of PC1 coinciding with the control (Fig. 4). The PLS-DA analysis resulted in a variance R^2 of 0.992 and a predictive ability $Q^2$0.953 indicating a good model.

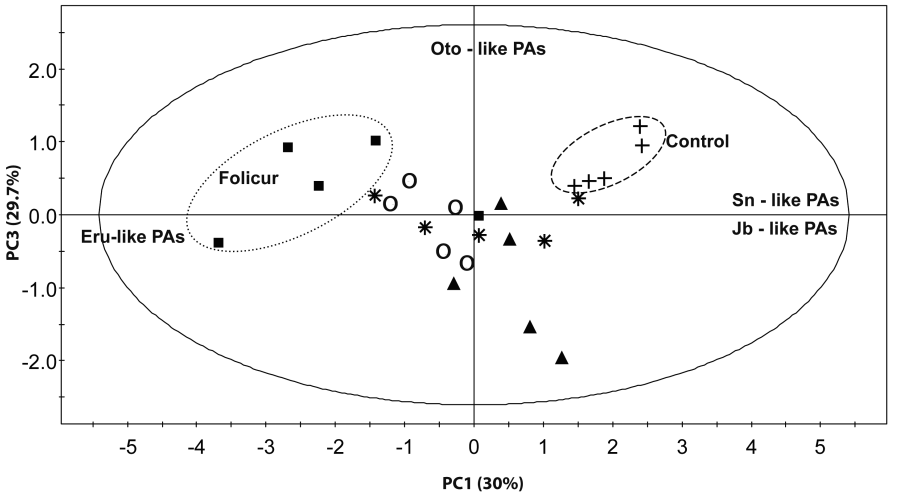

Fig 4. Score and loading plot of PLS-DA based on the relative concentrations of pyrrolizidine alkaloid (PA) types measured by LC/MS/MS in F2 hybrids of *Jacobaea aquatica* and *Jacobaea vulgaris* after fungicide treatment. The ellipse represents the Hotelling T2 with a 95% confidence interval. Symbols depict: starting material (▲); control (+); Folicur treatment (■); Pronto Plus treatment (○); Switch treatment (*).

NMR Metabolomics. Metabolites identified in the plant extracts of control and fungicide treatments included sugars, amino acids, organic acids, and phenylpropanoids. The identification of metabolites was based on NMR spectra of known compounds acquired in previous studies on *Jacobaea* plants (Leiss et al., 2009; Nuringtyas et al., 2012) and from comparison with our in house NMR spectra database (Kim et al., 2010).

The metabolite profiles based on the ^{1}H NMR spectra of the different fungicide treatments were compared with the control and starting material using PLS-DA. The PLS-DA score plot explained 66% of the variation and showed a clear separation of the starting material from all treatments (Fig. 5a). Control and fungicide treatments were clustered together in the negative quadrant of PC1. The loading plot showed that amino acids such as alanine and threonine as well as organic acids like malate and citrate were identified as important factors for the clustering of the starting

material. Chlorogenic acid and several primary metabolites like stachyose, mannitol, glutamate, adenosine, and fumarate were important factors for the clustering of the control and fungicide treatments (Fig. 5b).

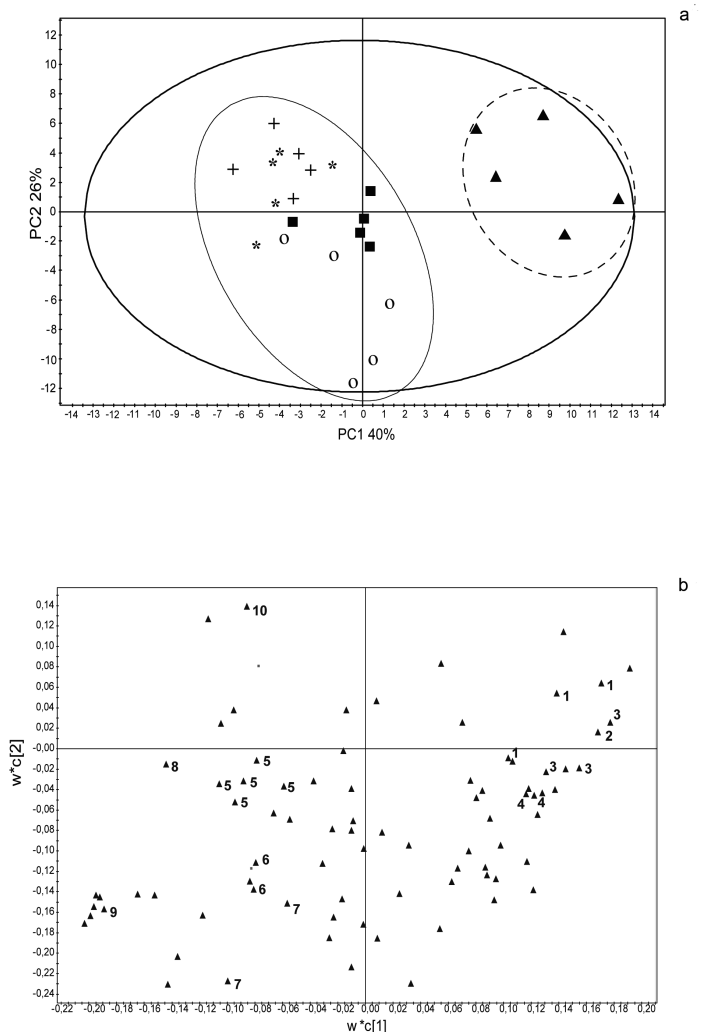

Fig 5. Score (A) and loading plot (B) of PLS-DA of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* after fungicide treatment based on 1H NMR spectra. The ellipse represents the Hotelling T2 with a 95% confidence interval. Symbols depict: starting material (▲); control (+); Folicur treatment (■); Pronto Plus treatment (○); Switch treatment (*). Malate(1); Threonine (2); Citrate (3); Alanine (4); Chlorogenic acid (5); Adenosine (6), Glutamate (7); Stachyose (8); Fumarate (9).;Mannitol (10).

Quantitative analysis of the compounds contributing to the separation of the starting material showed significantly higher levels of citrate (*ANOVA*, F=11.09, df= 4, P<0.0001), threonine (*ANOVA*, F=5.58, df= 4, P=0.003) and alanine (*ANOVA*, F=4.30, df= 4, P=0.011) in the starting material compared to the control (Fig. 6). Malate, which was identified in the loading plot of PLS-DA, showed a trend to accumulate in the starting material but failed to be significant. In

addition a higher level of inositol (*ANOVA*, F=3.30, df= 4, P=0.031) was observed in the starting material compared to the treatments. In contrast, significantly lower levels of adenosine (*ANOVA*, F=3.182, df= 4, P=0.036), fumarate (*ANOVA*, F=5.58, df= 4, P=0.003), stachyose (*ANOVA*, F=13.31, df= 4, P<0.0001), glutamate (*ANOVA*, F=4.170, df= 4, P=0.015), and phenylpropanoids such as chlorogenic acid (CGA) (*ANOVA*, F=14.49, df= 4, P<0.0001) and ferulic acid (FQA) (*ANOVA*, F=4.74, df= 4, P=0.07) were measured in the starting material compared to the control. Aside from that, the starting material showed a lower concentration of kaempferol analogues (*ANOVA*, F=3.52, df= 4, P=0.025) compared to the control. The level of mannitol was observed to be lower in the control compared to the starting material and fungicide treatments (*ANOVA*, F=3.52, df= 4, P=0.025).

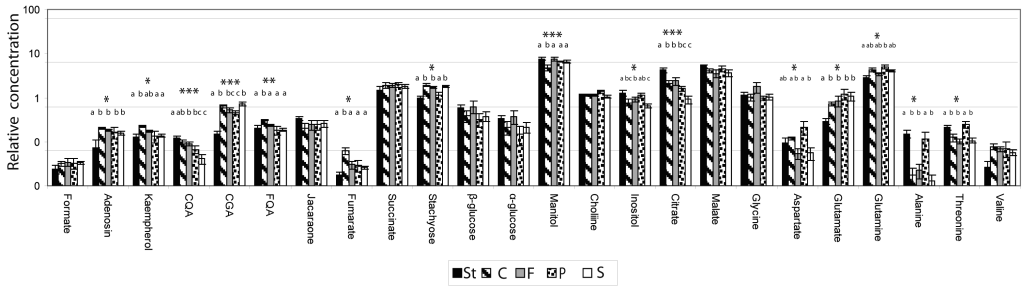

Fig. 6. Quantitfication of metabolites in F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* after fungicide treatment. Data present the mean of 5 replicates and the standard error. Symbols indicate: St = starting materials, C = control, F = Folicur treatment, P = Pronto Plus treatment, S = Switch. Data were analyzed using ANOVA. Different letters indicate means that are significantly different at * *P* <0.05, ** *P* <0.001, *** *P* <0.0001.

The fungicide-treated plants contained higher concentrations of mannitol but lower concentrations of fumarate and ferulic acid (FQA) than the control plants. Furthermore, the Folicur and Switch plants contained lower levels of the amino acid aspartate (ANOVA, F=4.23, df= 4, P=0.012) compared to the control. In addition to a low level of aspartate, the Switch plants were characterised by low levels of citrate and 5-*O*- caffeoylquinic acid (3-*O*-CQA) (ANOVA, F=8.63, df= 4, P<0.0001). The Pronto Plus plants were characterised by higher levels of the amino acids alanine and threonine while containing a lower level of stachyose.

DISCUSSION

The results of our study showed that *Jacobaea* plants, treated or not treated with fungicides, were able to synthesise PAs. This strongly indicates that endophytic fungi are not involved in PA synthesis. However, endophytic fungi may affect the total amount of PAs produced as well as their composition in the plant. This was observed in the Folicur plants, which contained low levels of total PAs as well as lower levels of senecionine- and jacobine-like PAs compared to the control and other fungicide treatments. A fungal endophyte closely related to Glomeromycota was identified in the Folicur plants at a relatively high concentration.

In all three fungicide treatments, Folicur, Pronto Plus and Switch, PAs were produced. There were no significant differences in the amount and composition of PAs synthesised in the Pronto Plus and Switch treatments and the control. In contrast, the Folicur-treated plants produced less PAs compared to the control and particularly Folicur plants contained less senecionine- and jacobine-like PAs. Only in the Folicur plants an endophyte could be detected. However, we should interpret this result carefully since it is generally accepted that endophytic fungi are ubiquitous and associated with more than 300,000 plant species (Hartley and Gange, 2009;Strobel and Daisy, 2003). Possibly, the concentration of endophytic fungi in the control was too low to be detected. The primers we used in this study are able to amplify the respective gene if the fungal DNA concentration is above 0.28 ng/ml. Fungal endophyte concentration can be as low as 0.1 ng/ml (Musgrave, 1984). However, our results give a strong indication that *de-novo* PA synthesis in *Jacobaea* plants is not depended on endophytes. Our finding is supported by a study reporting PA production of root cultures of *Jacobaea* under sterile conditions (Hartmann et al., 1989; Sander and Hartmann, 1989).

Both the β-tubulin and the ITS genes indicated that the endophyte detected in the Folicur plants showed a high homology with the fungal genus *Glomus*. The Glomeromycota division represents the arbuscularmycorrhizal fungi (AMF). It is known that the roots of *J. vulgaris* have an association with several AMF belonging to the Glomeromycota division (van de Voorde et al., 2010; Harley and Harley, 1987).This type of fungus falls indeed outside the spectrum of activity of tebuconazole, the active compound of Folicur, affecting Ascomycota and Basiodiomycota. Elimination of these fungi may have led to an advantage for the growth of *Glomus*.

Metabolomic analysis of the control and fungicide treatments showed no separation between the fungicide treatments and the control. This indicates that the changes in PA concentration and composition observed in the Folicur plants are a result of the interaction with the *Glomus* mycorrhizal fungus rather than the effect of fungicide treatment. Nothing is known about the role of *Glomus* influencing alkaloid composition. However, a recent study on *Glomus* mycorrhizae showed that the presence of these mycorrhizae reduced alkaloid concentrations in the grass *L. perenne* (Liu et al., 2011). The mechanism by which the *Glomus* mycorrhizae decrease alkaloid concentrations is unclear. The fungus may partly inhibit the *de-novo* PA synthesis or alternatively catabolise PAs. The concentration of senecionine *N*-oxide, the first product of the PA biosynthesis pathway, was significantly lower in the Folicur plants compared to the control and all other treatments. PA diversification is based on the transformation of senecionine *N*-oxide into other

PAs, except senecivernine (Hartmann and Dierich, 1998). The exact sequence of transformation from senecionine *N*-oxide into its derivatives is still under discussion. Two hypothetical biogenetic scenarios have been proposed. The first scenario suggests that formation of jacobine- and erucifoline-like PAs share the same biosynthetic route with the same enzyme being responsible for the transformation from senecionine *N*-oxide, while the otosenine-like PAs are formed independently as derivatives of senkirkine (Pelser et al., 2005) (Fig. S1.a). The second scenario proposes that jacobine- and erucifoline-like PAs are synthesised independently from each other while the otosenine-like PAs are derived from the jacobine ones (Pelser et al., 2005) (Fig. S1. b). The Folicur plants contained less senecionine- and jacobine-like PAs but erucifoline- and otosenine-like PAs were not affected. This indicates that the formation of jacobine-like PAs is independent from that of erucifoline-like PAs, while otosenine-like PAs are not derived from jacobine-ones. We thus propose that in this study PA diversification is a combination of both schemes (Fig. S1.c).

Pyrrolizidine Alkaloidss in the genus *Jacobaea* are known as constitutive defence compounds against pathogens (Joosten and van Veen, 2011) and generalist herbivores (Macel, 2011). Specifically jacobine-like PAs were reported to be involved in host plant resistance to the thrips *Frankliniella occidentalis* (Joosten, 2012; Cheng, et al., 2011; Leiss et al., 2009). In contrast specialist herbivores such as the Cinnabar moth (*Tyriajacobeae*) can use PAs as oviposition cues (Cheng, 2012; Macel and Vrieling, 2003). Jacobine chemotypes of *J. vulgaris* suffered less damage by generalist pathogens and insects in comparison to damage by specialist ones (Macel and Klinkhamer, 2010). An endophyte lowering the levels of total PAs in general and of jacobine-like ones specifically, may, therefore, affect plant defence against generalist and specialists. So far, the mycorrhizal fungus *Glomus mosseae* has been reported to reduce the beneficial effect of *Neotyphodium lolii*, a foliar endophytic fungus present in *L. perenne* on deterrence of the Noctuid moth, *Phlogophora meticulosa* (Vicari et al., 2002).

CONCLUSION

The *de-novo* PA synthesis in *Jacobaea* plants does not seem to be depended on endophytes. However, endophytes, as part of the biotic environment may influence the amount and composition of PAs produced. In this way endophytes may increase the variation of PAs in *Jacobaea* plants possibly leading to consequences in plant defence against generalist and specialist pathogens and herbivores.

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Supplementary data Chapter 2

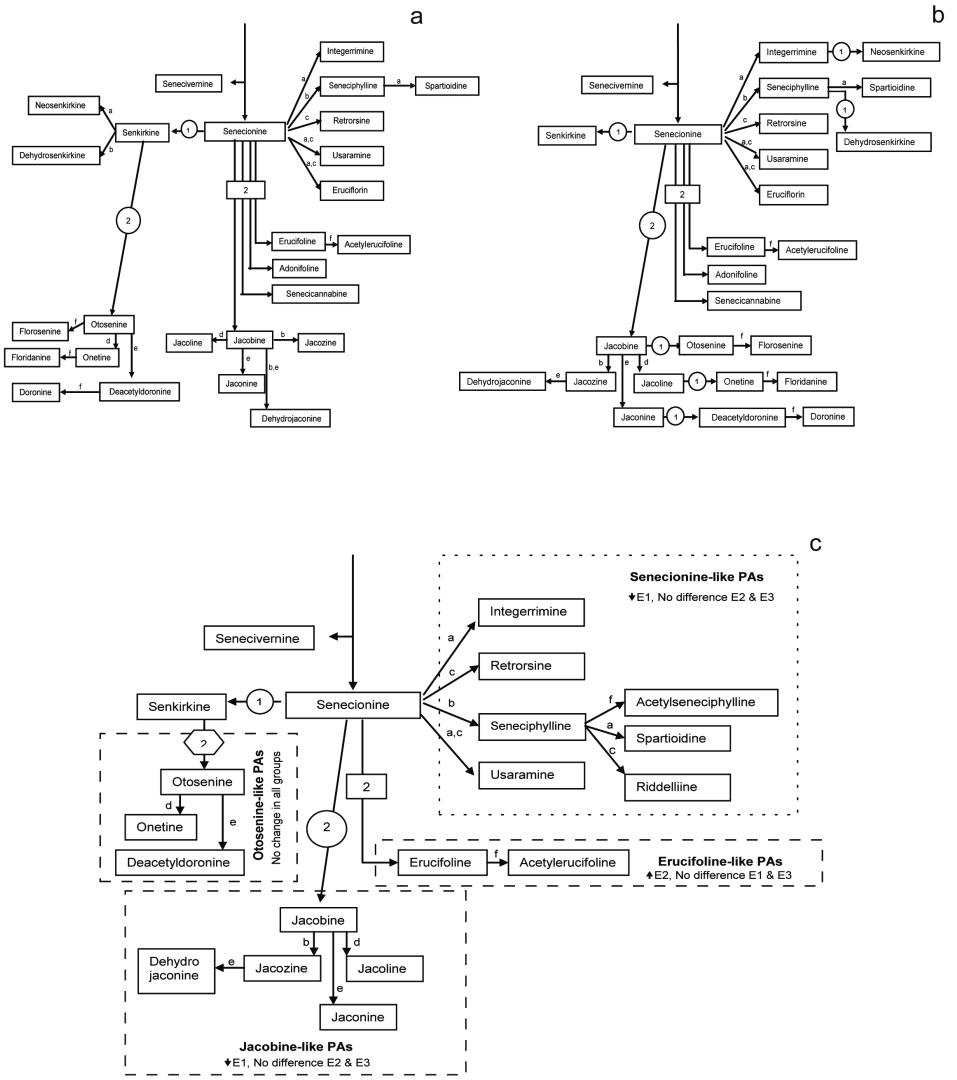

Fig S.1. Putative biosynthetic pathways for the diversification of PAs in the Jacobaea section: With the exception of senecivernine, senecionine is the common precursor of all other PAs. A and B are proposed by Pelser et al. (2005). A. Senkirkine is assumed to be a common precursor of all otonecine derivates; B. Otonecine derivates originate independently from the respective retronecine derivatives. In this case Jacobine- and erucifoline-like PAs are independent from each other. C. Combination of A and B based on the results of our fungicide treatments. The jacobine- and erucifoline-like PAs are independent from each other and the otonecine-like PAs derive from senkirkine. Two main reactions occur: conversion of retronecine to otonecine (reaction 1) and site-specific epoxide formation (reaction 2). Further structural diversification requires six simple one-step reactions marked by letters a-f: a= Z/E isomerization at C20; b= 13, 19-dehydrogenation; c = site-specific hydroxylations; d = hydrolysis of 15,20-epoxide; e = chlorolysis of 15,20-epoxide; f = site-specific O-acetylations.

Table S-1. ^{1}H chemical shifts (d) and coupling constants (Hz) in metabolites of F2 hybrids of *Jacobaea vulgaris* and *Jacobaea aquatica* identified by 1D and 2D NMR spectra in KH_2PO_4 and $MeOD_4$

No	Compounds	Chemical shifts (ppm) and coupling constants (Hz)
1	Adenine	d 8.33 (s), d 8.20 (s)
2	Alanine	d 1.47 (d, J = 7.2 Hz)
3	Aspartate	2.80 (m), d 3.80 (m)
4	3-*O*-caffeoyl quinic acid	d 5.43 (d, J = 5.6 Hz, 3.1 Hz), d 6.35(d, J = 15.9 Hz), d 7.60 (d, J = 15.9 Hz)
5	Chlorogenic acid (5-*O*-caffeoyl quinic acid)	d 5.42 (ddd, J = 10,8 Hz, 9.8 Hz, 5.6 Hz), d 6.28(d, J = 15.9 Hz), d 6.78 (d, J = 8.62 Hz), d 6.95 (dd, J = 8.21 Hz, 1.9 Hz), d 7.05 (d, J = 1.9 Hz), d 7.57 (d, J = 15.9 Hz)
6	Choline	d 3,22 (s)
7	Citrate	d 2.74 (d, *J* = 17.6 Hz), δ 2.56 (d, *J* = 17.6 Hz)
8	Formate	d 8.49 (s)
9	Fumarate	d 6.58 (s)
10	Feruloyl quinic acid	d 5.57 (dt, J = 8.0 Hz, 3.1 Hz), d 6.39 (d, J = 15.9 Hz), d 7.62 (d, J = 15.9 Hz)
11	Glutamine	d 2.15 (m), d 2.47 (m)
12	Glutamate	d 2.07 (m), d 2.36 (m)
13	Glucose	d 4.48 (H-β, d, J = 7,9 Hz), d 5.11 (H-α, d, J = 3,85 Hz)
14	Glycerol	d 3.50 (m), d 3.60 (m)
15	Inositol	d 3.15 (t, J = 9.27 Hz), d 3.43 (dd, J = 2.79 Hz, 9.78 Hz), d 3.96 (t, J = 2.67 Hz)
16	Jacaranone	d 6.16 (d, J = 9.6 Hz), d 7.05 (d, J = 9.6 Hz)
17	Kaempferol analogues	d 7.12 (d, J = 8 Hz), 8.01 (d, J = 8 Hz)
18	Mannitol	d 3.82 (d, J = 3.0 Hz)
19	Malate	d 4.34 (dd, J = 6.6, 4.7Hz), d 2.68 (dd, J = 16.6, 6.6Hz), d 2.78 (dd, J = 16.6, 4.7Hz),
20	Stachyose	d 5.47 (d, J = 3.8 Hz)
21	Succinate	d 2.54 (s)
22	Sucrose	d 5.39 (d, J = 3.8 Hz), d 4.13 (d, J = 8.5 Hz)
23	Threonine	d 1.30 (d, J = 6.6 Hz)
24	Valine	d 1.00 (d, J = 6.8 Hz), d 1.04 (d, J = 6.8 Hz),