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Metabolic changes in *Euphorbia palusrtis* latex after fungal infection

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ABSTRACT

The variations of metabolic profile of the latex of wild-growing *Euphorbia palustris* was carried out using multivariate analysis of ^{1}H NMR spectral data. One population was infected with fungi *Fusarium sporotrichioides*, *Fusarium proliferatum* and *Alternaria alternata*, while the other consisted of healthy plant species. The non-polar metabolites of latex extracts such as benzoyl ingenol-laurate, amyrin decadienoate esters, *cis*-1,4-polyisoprene, and 24-methylenecycloartanol were identified using ^{1}H and 2D NMR spectra. Principal component analysis of ^{1}H NMR data provided a clear discrimination between the latex of infected and healthy plants. Minimum inhibitory concentration and minimum fungicidal concentration values of the latex extracts of healthy and infected plants were determined. The latex of infected plants was found to contain higher levels of benzoyl ingenol-laurate and 24-methylenecycloartanol, of which concentrations were strongly correlated with the antifungal activities of the latex.

1. Introduction

The daily life of plants is greatly influenced by a wide range of biotic and abiotic factors. For many physiological purposes, e.g. defending against natural enemies or production of a certain compounds by sharing biosynthetic pathway in part plants are communicated with other organisms for their survival and all the organs of a plant (roots, leaves, and flowers) have a high level of microorganisms such as bacteria and fungi and especially moulds, that come from soil and air. It has been shown that leaves and aerial parts of the plants, especially young ones, due to their high humidity and roots and flowers, are more prone to infections than other parts such as fruits, seeds and bark (Bugno et al., 2006). Insects also, also contribute to an easier penetration of microorganisms in the plant tissue due to the mechanical damage.

A particular problem is the contamination of plants with fungi, especially moulds, because under a certain conditions, some of the ubiquitous fungal species can secrete mycotoxins that are toxic metabolites that have powerful mutagenic, carcinogenic, neurotoxic, nephrotoxic and/or immunosuppressive activities (Höhler, 2000; Hashem and Alamri, 2010). Fungi are ominipresent in nature and their spores can be found in the atmosphere even at high altitudes carried and scattered by the wind, insects and other animals (Kungulovski et al., 2011).

Recent studies have shown that fungal plant pathogens can be controlled by plant secondary metabolites and there are a number of plant extracts and essential oils that show antifungal activity against a wide range of fungi. There is thus great interest in their potential use as biofungicides within the viable pest control strategy known as biocontrol (Pal and Gardener, 2006).

Since plants are immobile organisms lacking the sophisticated immune system of animals and humans, they have developed their own defence system against pathogens and predators. This system can be roughly classified into two types: constitutive and inducible. The constitutive defences are present before pathogen infection, and plants may contain significant amounts of constitutive secondary metabolites including phenolic compounds, terpenoids and steroids, which can be toxic to invading organisms. But plants may also activate the production of certain defensive chemicals upon pathogen infection. This constitutes the inducible system and compounds are produced and accumulated after the specific recognition of an invading organism (Zeng, 2006). The examples of

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such compounds are phytoalexins, low molecular weight secondary metabolites with a wide range of chemical structures (Bennett and Wallsgrove, 1994). Apart from the production of phytoalexins, there are other types of resistance responses to attempted infections. For example, some plants can trap invading microbes with deposits of cell wall compounds such as lignin, callose, silicon, cellulose and glycoproteins. (Darvill and Albersheim, 1984; Abdel-Farid et al., 2009).

The Euphorbiaceae family is one of the most widespread plant families containing about 300 genera and more than 8000 species. The genus *Euphorbia* is the largest genus in the family with more than 2000 species sub-divided into several subgenera and sections. The native geographical distribution of *Euphorbia palustris* L. (Euphorbiaceae) (marsh spurge or marsh *euphorbia*), the species studied in this case, occupies a large region limited by the north of Spain, south Scandinavia, northwestern Kazakhstan, and southeast Turkey (Wärner et al., 2011).

Plants in the family Euphorbiaceae are well known for the chemical diversity of their terpenoid constituents. The largest number of compounds are diterpenoids with various structures such as jatrophanes, lathyranes, tiglianes, ingenanes, myrsinols, etc., all of which exhibit antimicrobial, antifungal, antiproliferation, cytotoxic, and anti-inflammatory activity. Many of these plants are used in folk medicine throughout the world (Shi et al., 2008). On the other hand, some diterpenoids from the latex of *Euphorbia* species are well known as irritants and tumor promoting agents (Kedei et al., 2004; Hecker, 1977). However, clinical studies performed with some of the compounds revealed promising healing properties (Ramsay et al., 2011). Among these, ingenol mebutate has recently been approved by the Food and Drug Administration (FDA) for use in the treatment of actinic keratosis. This is an example of an unmodified natural product that was introduced into clinical practice, something that occurs very rarely (Vasas et al., 2012).

All the mentioned bioactive diterpenoids are found in the latex of *Euphorbia* species. Latex is a plant secretion that consists in an aqueous suspension or emulsion of various metabolites such as sugars, proteins, rubbers, resins, and essential oils. It is produced in about 40 families, and more than 20,000 plant species. The biological function of latex is a protection against herbivores and microorganisms and to seal off wounds (Lewinsohn, 1991). Kniep reported the first experimental evidence of defensive role of latex in 1905 after observing that damaged Euphorbiaceae plants that were not able to exude more latex were much more susceptible to slug attacks. The sticky rubber-like precursors containing latex, provides resistance against herbivores by gluing their mouthparts or trapping their body parts. Also, a rapid wound closure prevents the infection by pathogens (Konno, 2011). The presence of other latex constituents such as the hydrolytic active proteins, lysozyme and chitinase also contributes to the plant defense mechanism (Sytwala et al., 2015). There are also reports of the antimicrobial activity of *Euphorbia* latex (Van Deenen et al., 2011; Goyal et al., 2012; Sumathi et al., 2011). All these findings converge *Euphorbia* latex against the attack of microorganisms could be assumed. The objective of this work was to study the impact of fungal infection of the plant on the metabolic changes in *Euporbia* latex, and thus approve its defense role.

Wild-growing *Euphorbia palustris* and fungus *Fusarium sporotrichioides*, *F. proliferatum* and *Alternaria alternata* were used to investigate the metabolic profile differences of the infected and healthy plants latex. For this, data obtained from the ^{1}H NMR spectra of latex extracts of plants with and without fungal infection was submitted to multivariate analysis to discriminate metabolites. These compounds and the latex of healthy and infected plants were then tested for their antifungal activity.

2. Results

2.1. Identification of secondary metabolites

Non-polar metabolites were identified in latex extracts dissolved in $CDCl_3$ using ^{1}H and 2D NMR spectra including COSY, NOESY, TOCSY, HSQC, HMBC, and *J*-resolved spectra (Table 1, Fig. 1).

The well distinguishable signals in the ^{1}H NMR spectra at δ 8.02 dd ($J = 8.5, 1.5$ Hz) and 7.47 dd ($J = 8.5, 7.6$ Hz) were attributed to the benzoyl moiety of benzoyl ingenol-laurate (**1**).

The characteristic double bond signals of amyrin decadienoate esters (**2**–**5**) occurred in the range of δ 5.7–7.7. The remaining aliphatic chain protons were assigned according to the COSY and TOCSY correlations (Table 1). The attachment of decadienoate chain to a carbinol carbon bearing a proton resonating at δ 4.57 (*m*) of amyrin moiety was rationalized by the HMBC correlation of this proton with the decadienoate ester carbonyl (δ_C 167.0). The E/E and E/Z geometric isomers, as well as α- and β-amyrin chain isomers were easily distinguished by their characteristic chemical shifts of double bond protons (Table 1).

The structure of *cis*-1,4-polyisoprene (**6**) was elucidated after a one-step purification from the latex extract using a Sephadex LH-20 column eluted with 2% methanol in CH_2Cl_2. The ^{1}H and ^{13}C NMR data were in full agreement with those in literature (Spanò et al., 2012; Jassbi et al., 2004).

The double bond signals of 24-methylenecycloartanol (**7**) at δ 4.71 br s and 4.66 br s, H-3 at δ 3.28 dd (J = 11.4, 4.5 Hz), cyclopropane ring protons at δ 0.55 br d (J = 4.0 Hz), and 0.33 br d ($J = 4.0$ Hz) were easily recognizable, but all remaining signals in the region of 0.7–2.4 ppm were overlapped.

The presence of benzoyl Ingenol-laurate (**1**), amyrin decadienoate esters (**2**–**5**) and 24-methylenecycloartanol (**7**) was confirmed by comparison with the chemical shifts of reference compounds that had been isolated previously in our laboratory (Fig. 2). Their structure was elucidated using 2D NMR techniques (see supplemental material) and by comparison with the spectral data from literature. (Wang et al., 2002; Lee et al., 2010).

2.2. Discriminating metabolites in chloroform latex extracts

In the ^{1}H NMR spectra of the $CDCl_3$ latex extracts of plant with fungal infection, the presence of signals attributed to benzoyl ingenol-laurate were clearly visible. These signals were barely noticeable in the spectra of the extracts of healthy plants (Fig. 3).

After the visual inspection of the ^{1}H NMR spectra, multivariate data analysis was applied to understand the overall variability within the samples. Principal component analysis (PCA), an unsupervised dimension reduction method was used for this purpose. Before PCA, the data was preprocessed using binning with total intensity normalization and Pareto scaling.

The highest separation was obtained with the first principal component (PC1) axes, a four-component model explaining 94.6% of the variance (Fig. 4). The examination of the scores plot (PC1 versus PC2) showed that the latex from all plants species from the first and healthy species from the 2nd - 4th collections (groups 1 and 2) was clearly separated along the PC1 axis from the infected ones (group 3). The latex from plants in groups 1 and 2 showed positive PC1 values, while those from group 3 showed negative PC1 values. In order to determine which variables were responsible for the separation in the PCA scores, the PC1 loading plot was examined. This showed large positive values of the signals at δ 5.12, 2.04, and 1.68 corresponding to *cis*-1,4-polyisoprene. The negative PC1 loadings of the signals at δ 8.02, 7.59, 7.47, 6.10, and 6.02, were attributed to benzoyl ingenol-laurate, and signals at δ 3.28, 0.55, and 0.33 to 24-methylenecycloartanol. The samples from the first