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Metabolomic analysis of isonitrosoacetophenone-induced perturbations in phenolic metabolism of *Nicotiana tabacum* cells

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ABSTRACT

Plants have developed biochemical and molecular responses to adapt to different stress environments. One of the characteristics of the multi-component defence response is the production of defence-related metabolites. Plant defences can be triggered by various stimuli, including synthetic or naturally occurring molecules, especially those derived from pathogens. In the current study, *Nicotiana tabacum* cell suspensions were treated with isonitrosoacetophenone (INAP), a subcomponent of a plant-derived stress metabolite with anti-fungal and anti-oxidant properties, in order to investigate the effect thereof on cellular metabolism. Subsequent metabolomic-based analyses were employed to evaluate changes in the metabolome. UPLC–MS in conjunction with multivariate data analyses was found to be an appropriate approach to study the effect of chemical inducers like INAP on plant metabolism in this model system. Principal component analysis (PCA) indicated that INAP is capable of inducing time-dependent metabolic perturbations in the cultured cells. Orthogonal projection to latent structures discriminant analysis (OPLS-DA) revealed metabolites of which the levels are affected by INAP, and eight of these were tentatively annotated from the mass spectral data and online databases. These metabolites are known in the context of plant stress- and defence responses and include benzoic- or cinnamic acid derivatives that are either glycosylated or quinilated as well as flavonoid derivatives. The results indicate that INAP affects the shikimate-, phenylpropanoid- and flavonoid pathways, the products of which may subsequently lead to an anti-oxidant environment *in vivo*.

1. Introduction

Plants can specifically recognise pathogenic micro-organisms and respond by activating appropriate multi-component defence mechanisms (McDowell and Dangl, 2000; Boller and Felix, 2009). By virtue of not having an adaptive immunity, plants rely solely on innate immunity of which there are two types namely pre-formed- and induced immunity. Chemical synthesis is regarded as an essential component of plant defence, supporting both the production of chemical compounds with direct or indirect anti-microbial activities and structural modifications to cell wall structures (Dixon et al., 1994; Hammond-Kosack and Jones, 1996; De Ascensao and Dubery, 2000). Plant metabolites with anti-microbial activity linked to preformed and induced immunity are known as phytoanticipins and phytoalexins, respectively (van Etten et al., 1994). Most of these anti-microbial metabolites include, amongst many, phenolic compounds, flavonoids, terpenoids, cyanogenic glycosides, hydroxamic acids and peptides (Bednarek and Osbourn, 2009). Since metabolites accumulate as the end products of cellular metabolism, their levels reflect the organism's ultimate response to biological or environmental changes (Fiehn, 2002). Metabolomic tools and metabolomic approaches have made significant contributions to the analysis of plant responses in e.g. wounding (Glauser et al., 2008), plant-pathogen interactions (Allwood et al., 2006, 2010), including pathogen-derived elicitors (Farag et al., 2008; Tugizimana et al., 2012, 2013).

As a consequence of innate immune responses, resistance can be induced in plants to avoid further spreading of the attacking pathogen. Uninfected sites become more resistant to subsequent infection, an adaptive phenomenon known as systemic acquired resistance (SAR) (Ryals et al., 1996). More recently, chemicals have also been employed to trigger a condition analogous to SAR, the most widely used being benzothiadiazole (BTH) with the trade name BION (Gatz and Lenk, 1998; Oostendorp et al., 2001; Dao et al., 2009). Promoters of genes with direct activity towards plant defence responses have been shown to respond to different types of chemical inducers (Görlach et al., 1996; Gatz, 1997). Although BTH is the most researched chemical inducer of plant defence, other molecules are also known to exhibit this activity. These include, amongst others, β-aminobutyric acid (BABA) (Jakab et al., 2001), methyl-2,6-dichloroisonicotinic acid (INA) (Métraux et al.,

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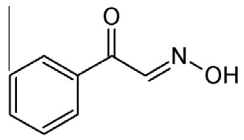

Fig. 1. The chemical structure of isonitrosoacetophenone (INAP).

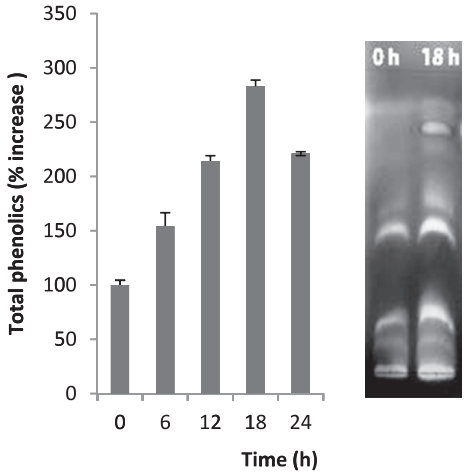

Fig. 2. Graphical representation of time-dependent (0–24 h) accumulation of total phenolics in INAP-treated tobacco cells. All samples are reported relative to the untreated control which was set as 100%. Three independent experiments (biological repeats) were conducted and the error bars represent the standard deviations. The insert is a TLC-DPPH assay showing the separation of antioxidant molecules appearing as white spots against a gray background.

1991), azelaic acid (Jung et al., 2009) and more recently, riboflavin (Liu et al., 2010a). The mechanism of action of these molecules is in most cases not well documented. Metabolomics can identify clusters of compounds to serve not only as a base in the search of novel defence compounds, but also as signatory bio-markers for the characterization of the plants' defensive state.

Dubery et al. (1999) reported the accumulation of an oxime-containing stress metabolite and phytoalexin, (4-(3-methyl-2-butenoxy)-isonitrosoacetophenone or citaldoxime) in citrus peel undergoing oxidative stress due to gamma radiation treatment. This novel compound was reported to exhibit phytoalexin, antioxidant and radical scavenging activities (Dubery et al., 1999). Although oxime functional groups are rare in natural products, they occur in a variety of phyla, e.g. sponges, bacteria, fungi, and plants (Almeida et al., 2011). In plants, oximes are known to be intermediates of a range of metabolic pathways (e.g. nitriles, cyanogenic glycosides, glucosinolates, etc.) subject to controls that result in variation in both the type and amount of end product formed (Mahadevan, 1973). In the context of plant defence and stress responses, aldoximes are intermediates or precursors during the biosynthesis of glucosinolates and cyanogenic glycosides, two classes of molecules that play vital roles during plant-herbivore interactions (Moller, 2010).

In the current study, isonitrosoacetophenone (INAP, or 2-keto-2-phenyl-acetaldoxime) (Fig. 1), a compound structurally similar to 4-(3-methyl-2-butenoxy)-isonitrosoacetophenone, was used to investigate induced metabolic changes in tobacco cell suspensions. Ultra-performance liquid chromatography coupled to mass spectrometry (UPLC–MS) was used for measuring the levels of physiological metabolites affected by INAP treatment, and principal component analysis (PCA) and orthogonal projection to latent structures discriminant analysis (OPLS-DA) of the UPLC–MS data discriminated between the metabolite content of untreated (control) and INAP-treated tobacco cell suspensions. The findings

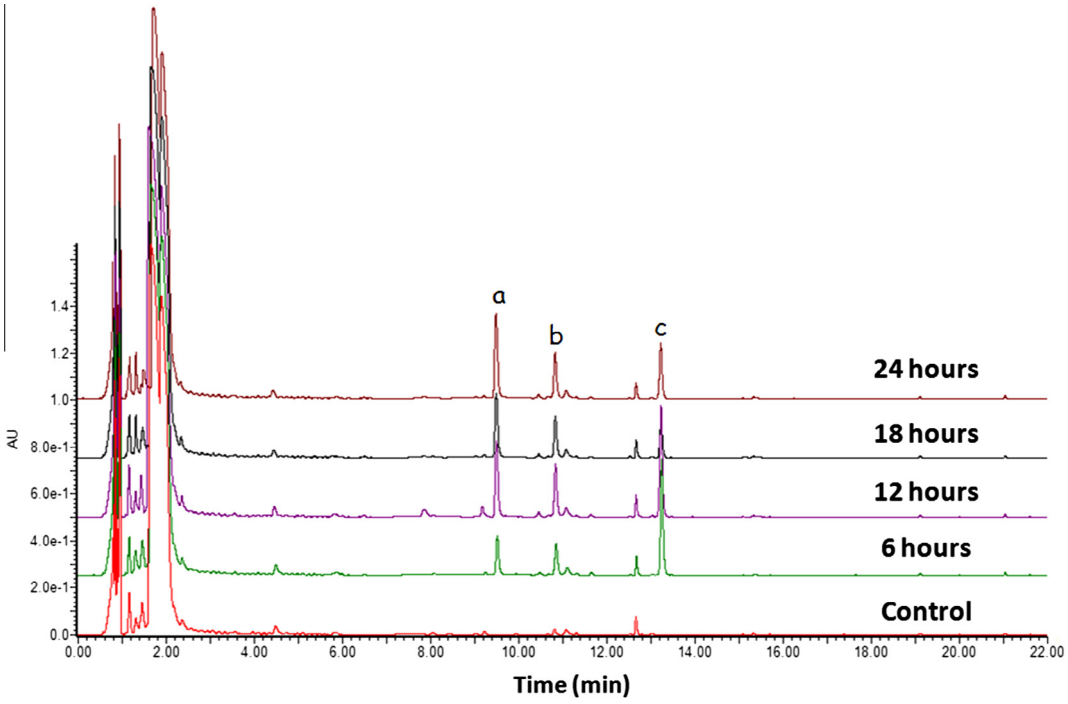

Fig. 3. Comparison of representative UPLC-PDA base peak intensity (BPI) chromatograms of tobacco cell suspension samples treated with INAP for different time intervals. Peak (a) represents molecules **3** and **4**, peak (b) represents INAP-biotransformed product (Madala et al., 2012) and peak (c) represents residual INAP.