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PhD Thesis

Camilla Badiali

Response of root cultures and *in vitro*-grown plantlets systems of *Hypericum perforatum* L. to biotic and abiotic stress

Tutor

Prof. Alessio Valletta

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Contents

Abbreviations chapter 1

13-HPOT	13-hydroperoxylinoleic acid
AOC	allene oxide cyclase
AOS	allene oxide synthase
APX	ascorbate peroxidase
As	arsenic
BPS	benzophenone synthase
Cd	cadmium
CHIT	chitosan
CHS	chalcone synthase
COS	chitosan oligosaccharides
ET	ethylene
ETC	electron transport chains
ETI	effector-triggered immunity
ETS	effector-triggered susceptibility
GR	glutathione reductase
HPLC	high performance liquid chromatography
HR	hypersensitive response
IAA	indole acetic acid
IBA	indole butyric acid
ICS	isochorismate synthase
ISR	induced systemic resistance
JA	jasmonate
JMT	JA carboxyl methyltransferase
LOX	lipoxygenases
MAMP	microbial-associated molecular pattern
MAO	monoamine oxidase
MAPK	mitogen-activated protein kinases
MeJA	methyl jasmonate
MeSA	methyl salicylate
NO	nitric oxide

OPR	12-oxo-phytodienoic acid reductase
PAL	phenylalanine ammonia lyase
PAMP	pathogen-associated molecular
PCD	programmed cell death
PIs	proteinase inhibitors
PR	pathogenesis-related proteins
PRR	pattern recognition receptors
PTI	PAMP-triggered immunity
ROS	reactive oxygen species
SA	salicylic acid
SAG	SA O-β-glucoside
SAGT	SA glucosyltransferase
SAMT	SA methyl transferase
SAPB2	MeSA methyl esterase
SAR	systemic acquired resistance
SOD	superoxide dismutases

1. Introduction

1.1 *Hypericum perforatum*

The genus *Hypericum*, the most numerous of the nine genera of Hypericaceae family (Stevens 2007; APG III 2009), includes almost 470 species of herbs, shrubs and small trees. It is distributed in Eurasia (>230 species), North and South America (c. 40 and >130 species respectively), Southeast Asia (c. 47) and Africa (c. 40 species). Typical habitats are rocky, sometimes calcareous, and dry to moist grasslands or acidic fens and shallow swamps. In the tropics it is generally confined to high elevation habitats (Robson 2003).

The most studied species of the genus is *Hypericum perforatum*, a medicinal plant used since antiquity with a broad range of pharmacological activities.

H. perforatum is commonly known as St. John's wort because of its blooming period which occurs at the end of June (June 24th is St. John's day). Christians believed that *H. perforatum* kept evil spirits away, for this reason on St. John's day they used to burn plants to purify the air and ensure good crop harvest, indeed the origin of the genus name "*Hypericum*" comes from Greek words *hyper* (over) and *eikon* (image, in the sense of ghost) because people believed in its exorcistical properties. The specific name "*perforatum*" refers to leaves that seem to be perforated due to the presence of secretory structures.

H. perforatum (Fig.1) is a completely glabrous herbaceous plant species, characterized by an erect stem, 30 to 100 cm long and branched in the upper section; the leaves (Fig. 1B) are yellow-green in color, opposite, shortly petiolate; yellow flowers (Fig. 1A) are numerous, forming a broadly paniculate, almost corymbose, inflorescence with numerous stamens free or into three bundles at the base. The ovary is superior and ovoidal with three widely divergent styles, flowers have five sepals and five petals with dark glands along the margins; the fruit (Fig. 1C) is a small septate capsule; seeds (Fig. 1D) are 1 mm long cylindric, brown, with a pitted or finely patterned surface (Bombardelli and Morazzoni 1995).

An anatomical characteristic of *H. perforatum* is the presence of two types of secretory structures on stems, leaves and flowers: translucent glands (Fig. 1B, E), which are colorless and accumulate hyperforin and essential oils, are localized all over the leaf lamina; dark glands (Fig. 1B, F), which are dark red and produce hypericin and its derivatives, are distributed on stems, leaves, flowers and anthers.

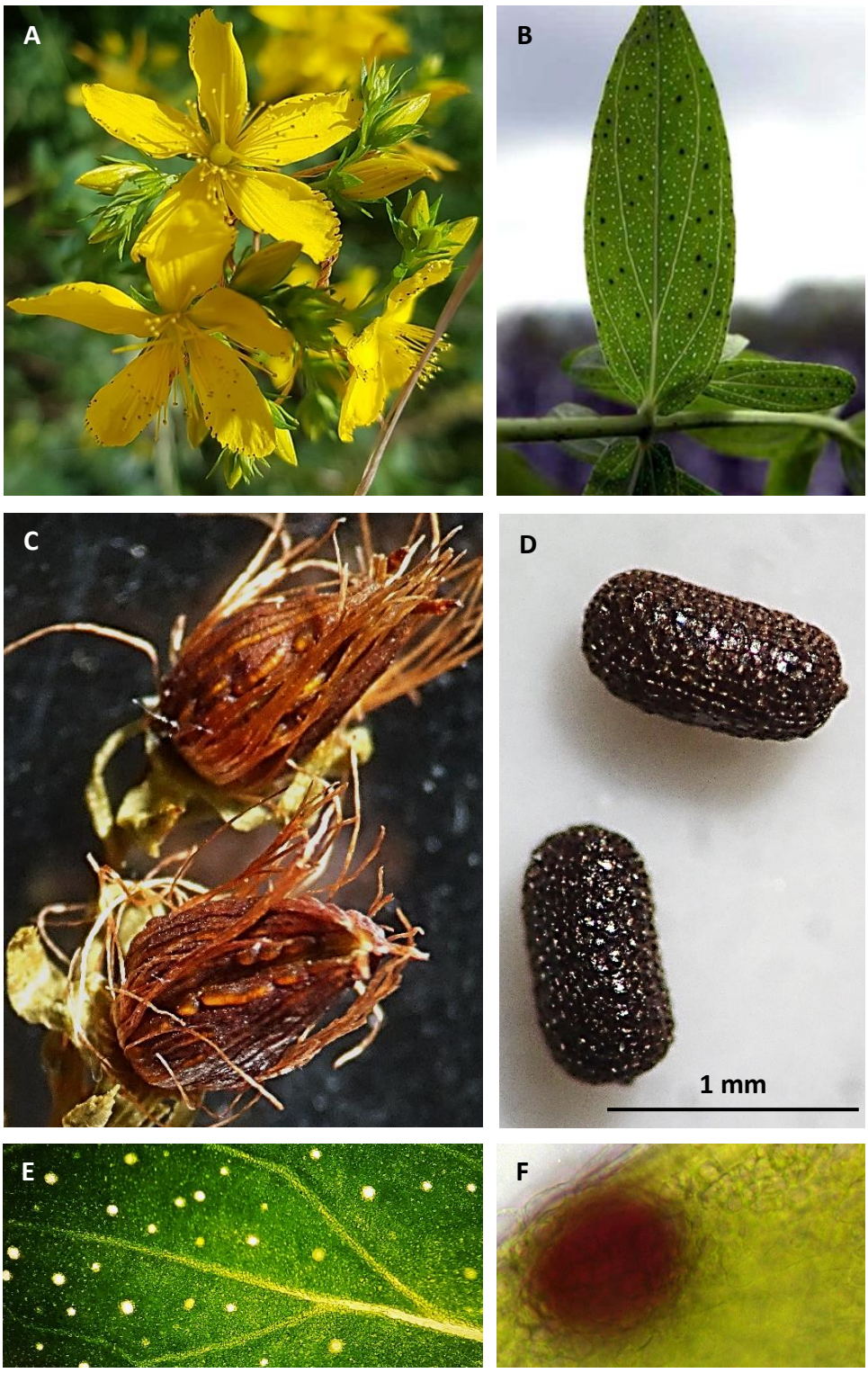

Fig. 1 ***Hypericum perforatum*****.** A) Flowers with numerous stamens; B) leaves showing dark and translucent glands; C) fruits, capsules; D) seeds; E) translucent glands; F) dark gland.

1.2 Traditional use, pharmacological activity and clinical properties of *H. perforatum*

The properties of *H. perforatum* have been known for centuries and its use in folk medicine has an ancient tradition. The Europeans have used it in order to treat a broad number of diseases, like anxiety, colds, depression, flue, hemorrhoids, womb muscle contractions during menstruation, skin irritations or infections, burns and wounds (Saddiqe et al. 2010 and literature cited therein). The oldest references about the use of *H. perforatum* come from the Greek botanist of the I century a.C., Dioscórides, the Roman student of the I century a.C., Plenius, and the Greek physician and father of medicine of the V century b.C., Hippocrates. In the XII century, Templars were the first to discover that *H. perforatum* was very useful for improving the mood of warriors forced to bed for months.

In last decades, *H. perforatum* has been studied primarily for the antidepressant activity of its extracts which have been widely sold in health food stores and pharmacies in Europe and the USA for the treatment of depression (Lecrubier et al. 2002; Kasper et al. 2006). Clinical studies found *H. perforatum* extracts to be efficacious in the treatment of mild to moderate depression (Kasper et al. 2010; Chen et al. 2011; Ng et al. 2017). The effectiveness of *H. perforatum* extracts has been also demonstrated in the treatment of somatoform disorders (Volz et al. 2002; Müller et al. 2004), obsessive-compulsive disorder (Kobac et al. 2005), anxiety (Singewald et al. 2004) and seasonal affective disorder (Wheatley 1999; Pjrek et al. 2005). Moreover, studies about other *H. perforatum* extracts properties have been conducted, including anti-Alzheimer (Hofrichter et al. 2016) wound-healing (Rao et al. 1991; Öztürk et al. 2007), anti-inflammatory (Kumar et al. 2001), antibacterial (Conforti et al. 2005), antifungal (Milosevic et al. 2007), and antiviral activities (Richer and Davies 1995).

1.3 Phytochemical content of aerial organs

The phytochemical content of leaves and flowers of *H. perforatum* has been deeply investigated because secondary metabolites with anti-depressant activity are produced and accumulate in the aerial organs of the plant (Mennini and Gobbi 2004). Hypericum preparations rich in bioactive secondary metabolites are obtained through hydroalcoholic extraction of the flowering tops and leaves; these are exploited above all for the treatment of depressive states, neurovegetative disorders and anxiety (Par 1.2).

Hypericins (naphthodianthrones) (Fig. 2A) and hyperforins (phloroglucinols) (Fig. 2B) are considered the main bioactive constituents of the aerial organs of *H. perforatum*, they are produced in black globules and translucent glands, respectively (Fig. 1E-F); hypericin was initially described as a monoamine oxidase (MAO) inhibitor (Suzuki et al. 1984), but later studies indicated that this effect was not clinically significant (Mennini and Gobbi 2004 and literature cited therein). In last decade the anti-depressant activity of hyperforin has been investigated, neglected at the beginning because of its instability in most organic solvents and fast air oxidation (Medina et al. 2002 and literature cited therein; Isacchi et al. 2007).
However, in addition to naphtodianthrones and phloroglucinols, several molecules have been characterized such as essential oils and volatile compounds (hydrocarbons, monoterpenes, sesquiterpenes) (Nahrstedt and Butterweck 1997; Crockett et al. 2010), flavonoids (flavonols, flavones, glycosides, biflavonoids, catechins), tannins and other phenols (caffeic, chlorogenic, *p*-coumaric, ferulic, *p*-hydroxybenzoic and vanillic acids) (Barnes et al. 2010 and literature cited therein).

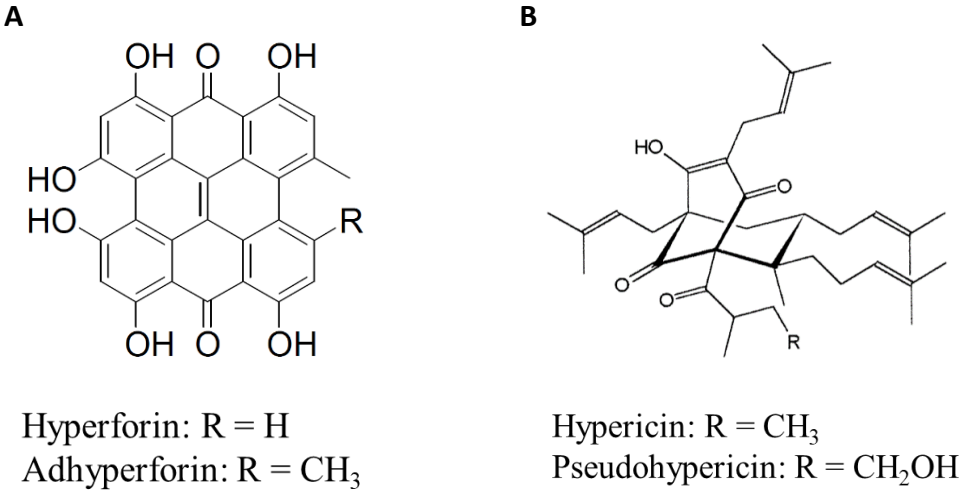

Fig. 2 Main bioactive secondary metabolites accumulated in *H. perforatum* shoots. Chemical structure of: A) hypericin and pseudohypericin; B) hyperforin and adhyperforin (Barnes et al. 2010).

1.4 Phytochemical content of roots

Although many therapeutic properties of aerial organ metabolites are known, little information is available on the chemical composition and biological activities of *H. perforatum* root extracts (Tocci et al. 2013; Simonetti et al. 2016).

The phytochemical content of St. John's wort root includes compounds also found in shoots such as phenolic acids (e.g. chlorogenic acid) and flavonoids (aglycon or glucoside flavonols like quercetin or hyperoside, respectively) (Cui et al. 2011) (Fig. 3A-C), but also xanthones, which are compounds specifically biosynthesized and accumulated in roots (Crockett et al. 2011).

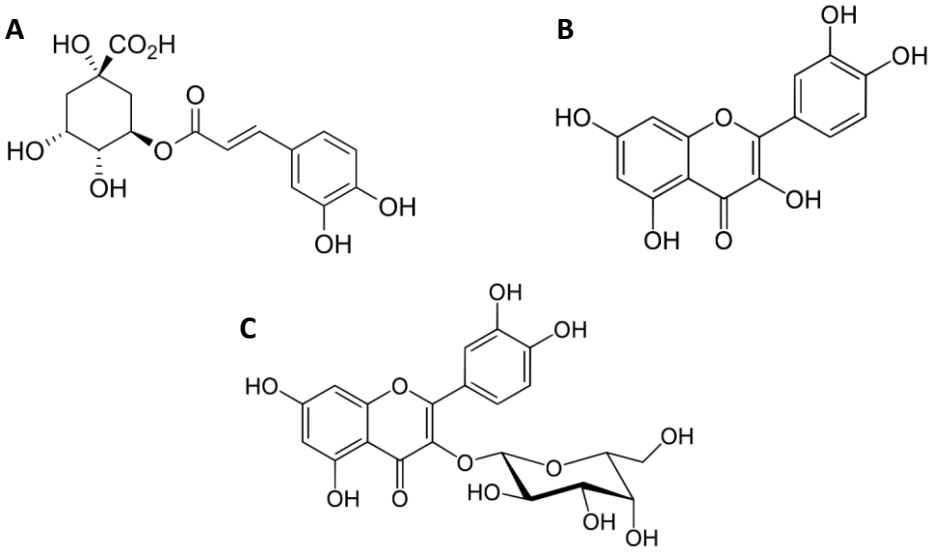

Fig. 3 Main polyphenols accumulated in *H. perforatum*. Chemical structures of: A) chlorogenic acid; B) of quercetin and C) hyperoside.

1.5 Structure and chemical properties of xanthones

Xanthones, from Greek "ξανθός" (xanthós) which means "yellow", are phenolic compounds with a limited distribution in plant kingdom. Xanthone (9*H*-xanthen-9-one) (Fig. 4) is the central core to which substituents are added to form different xanthones usually found as aglycones, O-glycosides and C-glycosides. They are produced mainly by plants, but also by some lichens, fungi and bacteria (Masters e Brase, 2012). Xanthones are typical secondary metabolites of plants belonging to the Hypericaceae family in particular of *Hypericum* species, but they are also synthesized in species belonging to Gentianaceae, Moraceae and Polygalaceae families (Negi et al. 2013).

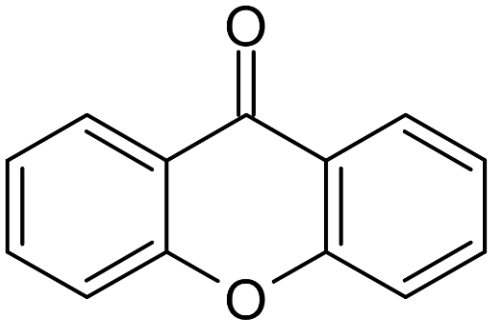

Fig. 4 Chemical structure of xanthone.

1.5.1 Xanthone biosynthesis

Exodermis and endodermis are the sites of xanthone biosynthesis in *H. perforatum* roots (Tocci et al. 2018); they are synthesized through the phenylpropanoid pathway which has different branches leading to many different compounds including simple phenylpropanoids, flavonoids, lignin and others (Demirkiran 2007).

Key enzymes regulate this pathway moving the metabolism depending on plant's needs. The phenylpropanoid pathway originates from phenylalanine, which is deaminated by phenylalanine ammonia lyase (PAL). This is one of the well-studied and characterized enzyme of plant secondary metabolism, it catalyzes the reaction which links primary metabolism (shikimate pathway) to secondary metabolism: phenylalanine deamination to *trans*-cinnamic acid (Fig. 5) with release of nitrogen in the form of ammonia. The biosynthetic pathway divides in two branches from *trans*-cinnamic acid to give xanthones or flavonoids. *p*-coumaroyl-CoA and malonyl-CoA are produced from *trans*-cinnamic acid and two enzymes act on these compounds respectively: chalcone synthase (CHS) and benzophenone synthase (BPS) (Liu et al. 2003; Vogt 2010).

CHS in presence of one molecule of *p*-coumaroyl-CoA and three of malonyl-CoA catalyzes consecutive decarboxylations and condensations leading to chalcone, the flavonoid precursor from which tannins, flavones, isoflavones, flavonols and anthocyanins are synthesized.

BPS, a transferase, in presence of one molecule of benzoyl-CoA and three of malonyl-CoA catalyses the reaction which leads to benzophenone, the precursor of xanthones.

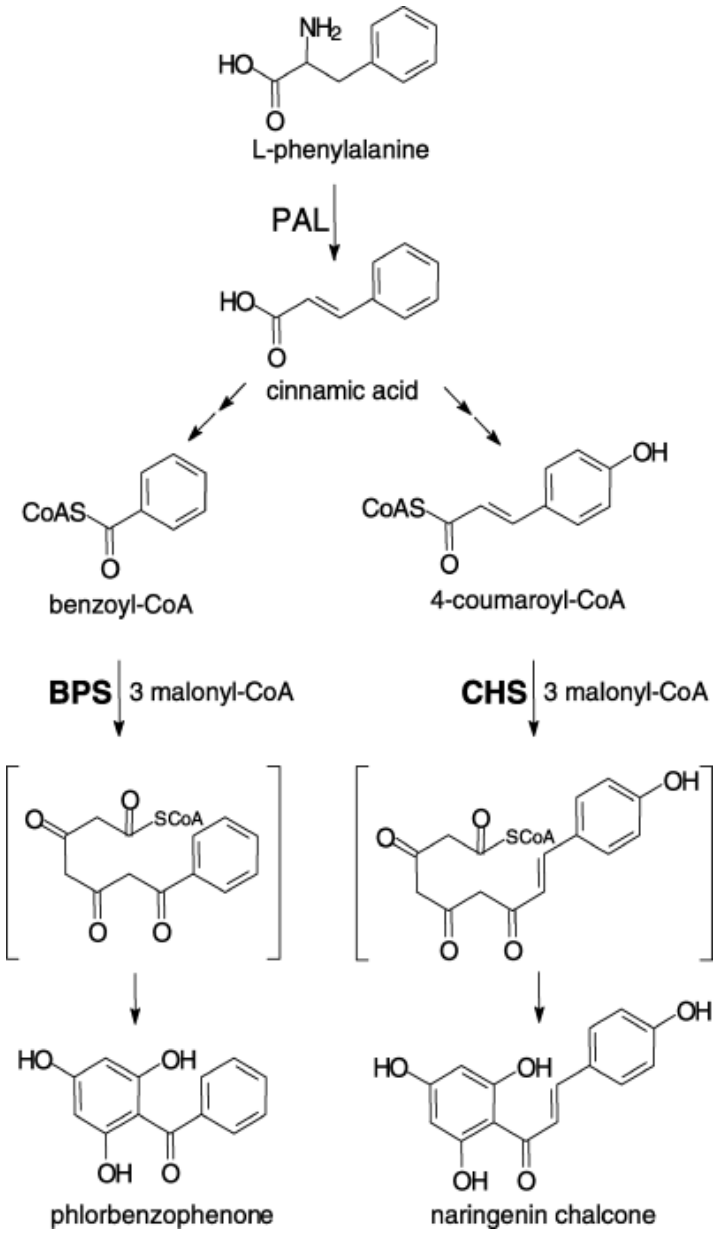

Fig. 5 Phenylpropanoid pathway. Branches leading to xanthone and flavonoid synthesis. PAL: phenylalanine ammonia lyase; CHS: chalcone synthase; BPS: benzophenone synthase (Liu et al. 2003).

1.5.2 Biological activities of xanthones in plants

Phenolic compounds are known to be molecules involved in protecting plants against microbial attacks (Ahuja et al. 2015); *in vitro* studies have shown that xanthones, as observed for other polyphenols, are constitutively biosynthesized, although their content may significantly increase in response to pathogenic attack (phytoalexins). An increase in xanthone accumulation in cell cultures of many plant species subjected to elicitor treatments

has been observed: *Centaurium erythraea* elicited with yeast extract and methyl jasmonate (MeJa) (Beerhues and Berger 1995); *H. perforatum* elicited with *Colletotrichum gloeosporioides* cell wall extracts, *Agrobaterium tumefaciens*, MeJa, salicylic acid (SA) and chitosan (CHIT) (Conceicao et al. 2006; Franklin et al. 2009; Tocci et al. 2010). Franklin and colleagues (2009) proposed a double role of xanthones in the defense response as antioxidant and antimicrobial compounds: *in vivo* xanthones on one hand constitute a powerful antioxidant system to protect the host cells from reactive oxygen species (ROS) and on the other hand have also the potential to act as phytoalexins against pathogenic microorganisms (Fig. 6).

In the last decades the inducible role of xanthones has been also investigated in organ cultures (Ishimaru et al. 1990; Vinterhalter et al. 2008; Tocci et al. 2011, 2012; Valletta et al. 2016). *In vitro* cultures of *H. perforatum* roots elicited with chitosan or chitosan oligosaccharides (which mimic a fungal attack) synthesized xanthones significantly increasing the concentration compared to non-treated samples (Tocci et al.2010, 2011; Brasili et al. 2014; Badiali et al. 2018).

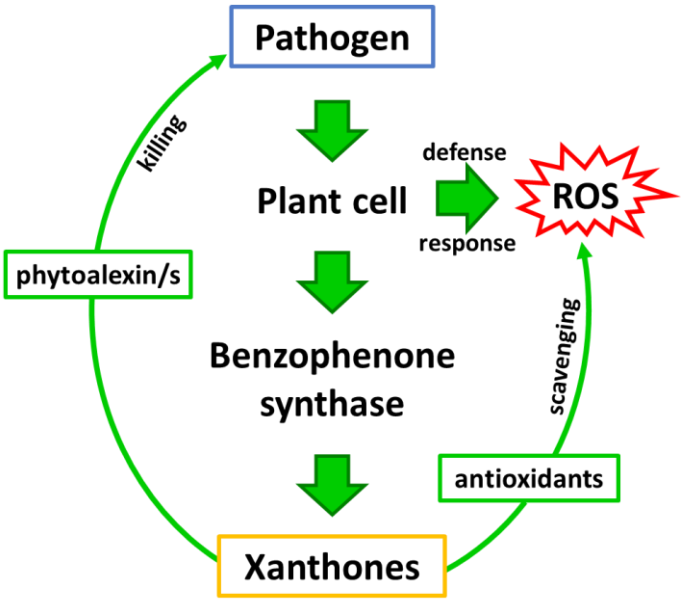

Fig. 6 Hypothetical role of xanthones in the plant pathogen-interaction. In the model proposed by Franklin et al. (2009) xanthones play dual function in plant cells during biotic stress: (1) as antioxidants to protect the cells from oxidative damage and (2) as phytoalexins to impair the pathogen growth (modified from Franklin et al. 2009).

1.5.3 Pharmacological activities of xanthones

The interest in studying xanthone pharmacological activity had its beginning in ‘60s when diuretic and cardiotonic effect of mangiferin, a compound belonging to this class, was demonstrated (Finnegan et al. 1968 and literature cited therein). Following studies led to the discovery that xanthones can reverse various types of tumor (Abe et al. 2003; Chen et al. 2004; Su et al. 2011; Núñez et al. 2016) and that they have antimutagenic and antiangiogenic activities (Mackeen et al. 2000; Pinto et al. 2003; Almanza et al. 2011; Núñez et al. 2016). Xanthones have also anti-inflammatory properties (Chung et al. 2002; Park et al. 2006; Chen et al. 2008), antioxidant activity (Panda et al. 2013), they are effective in the treatment of cardiovascular diseases (Ishiguro et al. 2002) and have an enzyme inhibitory activity against mono amine oxidase (MAO) enzyme which plays an important role in the regulation of some neurologically active amines (Ohishi et al, 2000; Gnerre et al. 2001; Urbain et al. 2008); the inhibitors of MAO are useful in the therapy of several neurodegenerative conditions, including Parkinson's disease and Alzheimer's disease, psychosis, depression and schizophrenia (Laban and Saadabadi 2019 and literature cited therein; Wang et al. 2019).
In the last decades antifungal, antibacterial and antiviral activities against human pathogens have attracted the attention of scientists because of the onset of resistance of pathogens to conventional antibiotics and antifungals (De Vita et al 2012). Xanthones, in particular prenylated and oxygenated xanthones, showed a significant antimicrobial activity against microorganisms pathogenic to humans (Fotie and Bohle, 2006; Tocci et al. 2011, 2012, 2013; Simonetti et al. 2016; Badiali et al. 2018) and plants (Cortez et al. 1998; Crockett et al. 2011).

1.6 Plant defense responses

Plants are sessile organisms in environments inhabited by living beings potentially dangerous to them (pathogens or phytophages) (biotic stress); moreover, they are subjected to stress caused by non-living factors depending on climate conditions (abiotic stress). For these reasons they had to evolve specific mechanisms to detect and consequently act against complex stress combinations, minimizing damage while conserving resources for growth and reproduction.

Plants have evolved different ways to perceive external attacks. As shown in Fig. 7, the recognition of microbial- or pathogen-associated molecular pattern (MAMP or PAMP) perceived by host encoded pattern recognition receptors (PRRs) leads to the PAMP-triggered immunity (PTI, called basal resistance). Successful pathogens secrete pathogen-encoded effector proteins which suppress PTI leading to the effector-triggered susceptibility (ETS), these factors are in turn recognized by plant resistance (R) proteins leading to the effector-triggered immunity (ETI). Natural selection drives pathogens to avoid ETI diversifying or acquiring new effectors.

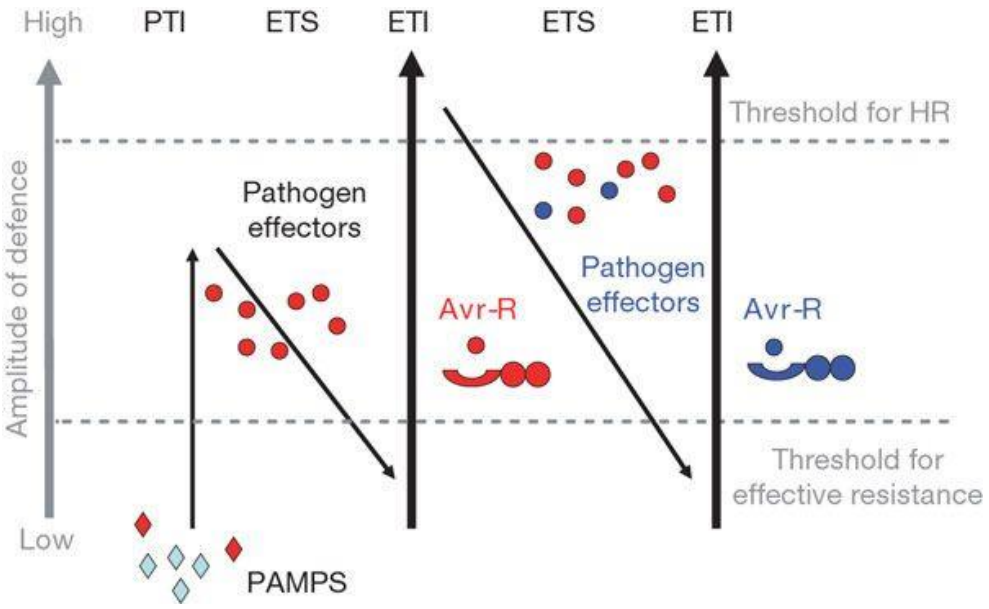

Fig. 7 "Zig-zag" model for plant immune system (Jones and Dangl 2006).

The typical manifestation of ETI, mostly against biotrophic pathogens and viruses (Glazebrook et al. 2005) is hypersensitive response (HR) (Chisholm et al. 2006; Bari et al. 2009). The latter consist in the developing of necrotic lesions at the pathogen entry site to prevent the invasion into plant tissues and to deprive the pathogen of nutrients. ETI usually causes the accumulation of reactive oxygen species (ROS) and nitric oxide (NO), and the activation of defense-related genes including those encoding pathogenesis-related (PR) proteins (Dempsey et al. 1999). Ion fluxes (in: Ca^{2+} and H^{+}; out: K^{+} and Cl^{-}) occur in cells next to the invasion site causing the production of toxic compounds formed by molecular oxygen reduction, such as superoxide anion (O_2^{-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH•); these reactive species start radical chain reactions involving a wide variety of organic substances leading to lipid peroxidation, enzymatic inactivation and nucleic acid

degradation (Lamb and Dixon 1997), contributing to apoptosis and acting directly against the pathogen. Moreover, ROS are involved in cell wall fortification causing proline-rich protein modification (Bradley et al. 1992), this adds to lignification and callose apposition to create a barrier against the invader. At the same time NO is produced. Both NO and ROS can act also as signaling molecules (although their long-distance action is unlikely). They appear to function in a positive feedback loop with SA and their production is necessary for HR activation (Fig. 8). NO induce SA accumulation and SA is required in NO defense signaling. H_2O_2 increases following a pathogen attack and activates SA synthesis; SA then cooperates with ROS generated during the second phase of the oxidative burst potentiating cell death and genes involved in plant defense. SA also induces an increase in H_2O_2 production, which in turn activates the synthesis of more SA and programmed cell death (PCD) in a self-amplifying loop which regulates defense responses (Dempsey et al. 1999; Durner et al. 1997; Overmyer et al. 2003).

The phytohormone SA has a prominent role in HR (Par. 1.7.1.4) activating non-expressor of PR genes 1 (NPR1) which in turn activates PR proteins; moreover, SA is also necessary for systemic acquired resistance (SAR) induction, probably acting via its volatile methyl ester methyl salicylate (MeSA) (Park et al 2007). Among PR proteins hydrolytic enzymes are the principal group, they attack the pathogen cell wall and degrade it. At last, phytoalexins are produced, secondary metabolites with a high toxicity against pathogens. A few hours to several days after HR, also in distant portions of the plant PR gene expression levels increase leading to the development of SAR, a long-term resistance to infection by a broad diversity of pathogens.

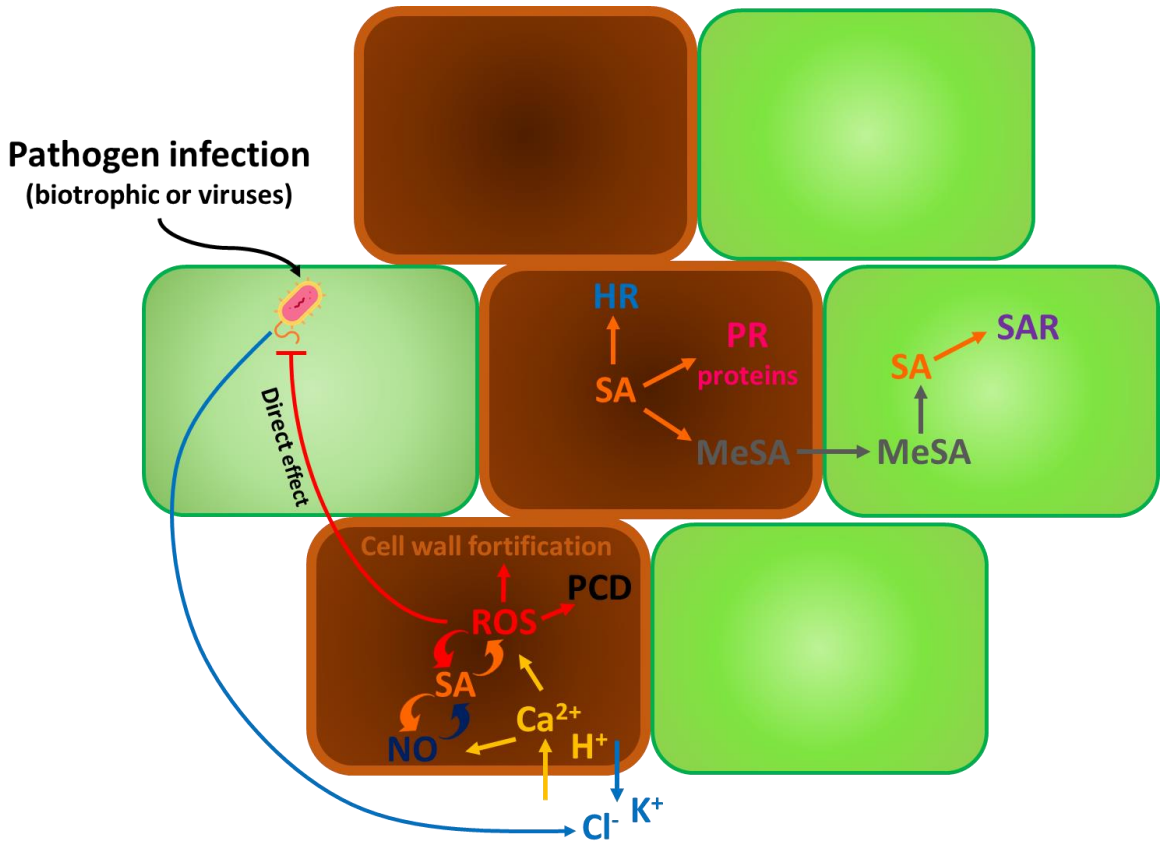

Fig. 8 Plant response against pathogen infection. SA: salicylic acid; ROS: reactive oxygen species; NO: nitric oxide; MeSA: methyl salicylate; PCD: programmed cell death; PR proteins: pathogen related proteins; HR: hypersensitive response; SAR: systemic acquired resistance.

Response against herbivore insects and necrotrophic pathogens involves other signalling molecules. The principal signalling pathway involved in plant defense responses against pests is the octadecanoid pathway which produces the phytohormones jasmonic acid (JA), methyl jasmonate (MeJA) and derivatives (Par. 1.7.1.3). This pathway is activated by pest oral secretions (Halitschke et al. 2001) and mechanical wounding stress leading to the release of systemin, a signal peptide of 18 amino acids which is cleaved from the C-terminal region of a 200-amino acid precursor protein called prosystemin; it induces a signal cascade leading to the activation of octadecanoid pathway for JA production which in turn causes the expression and the accumulation of defensive proteinase inhibitors (PIs), which play a defensive role by inhibiting the activity of digestive enzymes in the guts of insects (Sun et al. 2011) and induces the production of phytoalexins both locally and systemically.

Jasmonates, synergistically with ethylene (ET), are also involved in induced systemic resistance (ISR) activated by non-pathogenic microorganisms; this response alerts the plant and causes the establishment of an advanced state of preparation against pathogenic attacks

(priming for enhanced defense). This type of systemic defense does not include the production of SA or PR proteins (Hase et al. 2008; De Vleesschauwer et al. 2008; Segarra et al. 2009) (Fig. 9). The defense regulatory protein NPR1 is an important regulator in SA-dependent SAR and acts also in JA/ET-dependent ISR (Dong 2004; Pieterse and Van Loon 2004; Leon-Reyes et al. 2009) but its activation does not induce the expression of SA-responsive PR-genes. This suggests that NPR1 plays a key role in regulating and connecting different induced defense pathways (Dong 2004; Pieterse et al. 2009) via some factors (WRKY and MAPKs – mitogen activated protein kinase) involved in mediating the bidirectional antagonism between SA- and JA-mediated signaling (Li et al. 2004, 2006; Brodersen et al. 2006; Qiu et al. 2008) although synergism between these pathways has been observed (Mur et al. 2006).

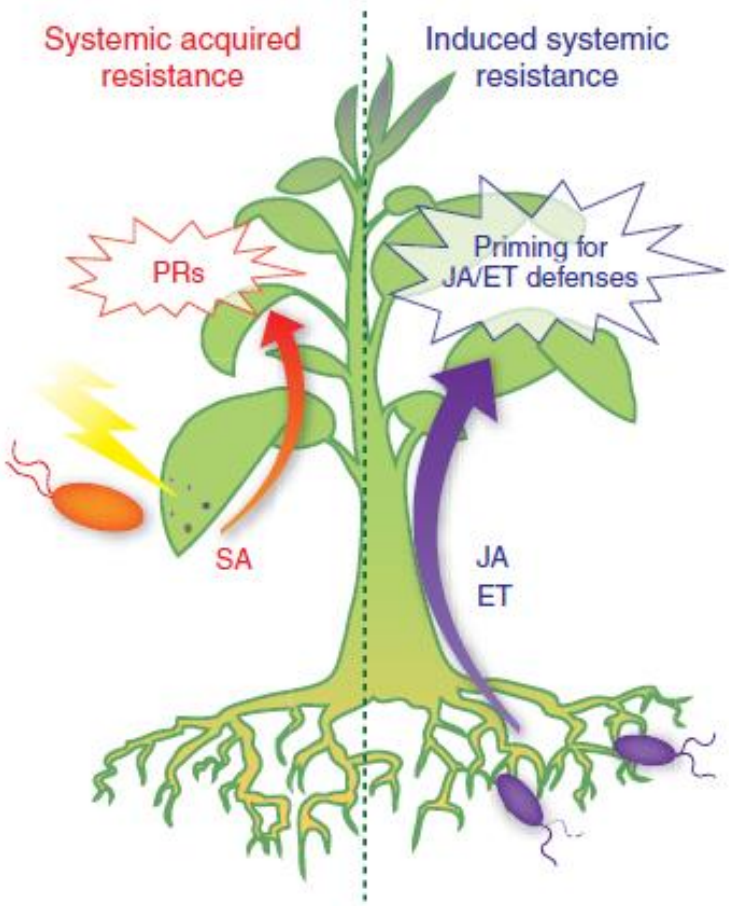

Fig. 9 Systemically induced immune responses (Pieterse et al. 2009).

1.7 Induction of responses in plant cultures: advantages of *in vitro* systems and elicitation techniques

The major source of bioactive compounds for medicine, food additives, pigments, insecticides, cosmetics, and fine chemicals has historically been the plant kingdom. However, the extraction of bioactive compounds directly from plants is often not efficient, the extract may contain only very small quantities, or its composition may vary with the season or the environment. Often the biosynthesis of some compounds is activated in specific stages of development or in response to stress, specific environmental conditions and nutrients availability. Also, the characteristics of the producer organism may limit the availability of the molecules of interest: the plant could be rare, could have a slow or a difficult growth, or could be a protected species (Verpoorte et al. 2002). To obtain qualitatively and quantitatively standardized extracts, plant biotechnology can represent a valuable alternative. This technology is advantageous compared to the conventional agricultural production because it is independent of geographical and seasonal variations and various environmental factors; it offers a defined production system; it allows to obtain a rapid production and an efficient recovery; it allows to use plants as biotransformers for the production of novel compounds from cheap precursors.

For these reasons, in the last decades many studies have focused on plant cell cultures as a possible method to produce plant secondary metabolites of commercial interest (Buitelaar and Tramper 1992; Lipsky 1992; Verpoorte et al. 1993, 1998; Su 1995). Many authors demonstrated that in undifferentiated calli and suspended cells of *H. perforatum* xanthones are the main secondary products accumulated (Dias et al. 1999; Dias et al. 2001; Pasqua et al. 2005; Conceicao et al. 2006; Mulinacci et al. 2008). However, for many of the desired compounds, the production from cell cultures is too low, not exploitable for applicative purposes. This is usually because some compounds require tissue differentiation to be correctly synthesized.

To increase the production of secondary metabolites (including xanthones) elicitors can be used. The elicitation is a technique commonly used in plant biotechnology to enhance secondary metabolite production (Zhao et al. 2005; Namdeo 2007).

Elicitation techniques have been applied to suspended cell cultures of *H. perforatum* (literature cited in Par. 1.5.2) and the xanthone biosynthesis was significantly stimulated but it was insufficient for a large-scale production. The use of elicitors on organ cultures allowed to obtain the highest xanthone production, the adventitious root cultures of *H. perforatum*

demonstrated to be a promising reliable way for production of pharmaceutically and nutraceutically important metabolites (literature cited in Par. 1.5.2).

1.7.1 Biotic stress

Biotic stress is stress caused by a living organism to another; plants are continuously exposed to biotic stress factors such as the attack by fungi, bacteria, viruses, nematodes and herbivores. Nonetheless, disease takes place only if the pathogen overcomes the diverse defense strategies that plants put in place against the invader. After the attack, plants activate signaling pathways to organize a response against pest, the response culminates in the production of secondary metabolites (Par. 1.6). The involved signal molecules can be used in plant biotechnology as elicitors to mimic the attack and consequently induce the biosynthesis of secondary metabolites of interest or to investigate plant responses.

1.7.1.1 Chitin and chitosan

Chitin is a linear polysaccharide composed by *N*-acetyl-D-glucosamine repeat units linked by β-(1 $\rightarrow$ 4) glycosidic bonds (Fig. 10A). It is the principal component of the exoskeleton of arthropods and fungal cell wall, and the second-most abundant polysaccharide on earth following plant cellulose. Plants do not contain chitin but possess enzymes to degrade it (chitinases), probably because of the coevolution with their fungal pathogens (Passarinho and de Vries 2002). Chitinases act both directly on fungi degrading their cell wall, and indirectly generating chitin fragments that are recognized as stress signals by plant cells (Boller 1995). Unfortunately, chitin has limited utility for human applications due to its low solubility.

Chitosan is obtained from a partial deacetylation of chitin (Fig. 10B) so it is an heteropolymer composed by *N*-acetyl-D-glucosamine and D-glucosamine, whose relative amount may vary resulting in different degrees of deacetylation, molecular weights, viscosities etc. (Raafat and Sahl 2009 and literature cited therein). Chitosan is more soluble than chitin, is biodegradable, atoxic and non-allergenic (Raafat and Sahl 2009 and literature cited therein). Among chitosan biological activities there are antimicrobial (Rebea et al. 2003; Eaton et al. 2008), antioxidant (Yen et al. 2008), and hypocholesterolemic activity

(Xia et al. 2011 and literature cited therein). Moreover, chitosan promotes plant growth (Chibu et al. 2000) and it was reported to induce plant defense responses via raising of cytosolic Ca^{2+}, activation of mitogen-activated protein kinases (MAPK), callose apposition, generation of ROS, hypersensitive response (HR), synthesis of abscisic acid (ABA), jasmonate, pathogenesis related proteins (PR) (Iriti and Faoro 2009 and literature cited therein) and phytoalexins (Fan et al. 2010; Sivanandhan et al. 2012; Sathiyabama et al. 2016) including xanthones in various species (Tocci et al. 2010; Krstić-Milošević et al. 2017). Despite many advantages, it has been reported that the use of chitosan at high concentrations (necessary for massive industrial production of secondary metabolites) has also inconveniences including the inhibitory effect on biomass growth *in vitro* and the irreversible morpho-anatomical alterations which make elicited biomass *in vitro* no longer usable for further production cycles (Brasili et al. 2014). Moreover, chitosan is insoluble in neutral water and other organic solvents, for this reason it is solubilized in water acidulated with acetic acid. This makes its use difficult in food and biomedical applications. Moreover, its use in basic research is also limited, at least in *H. perforatum*, as it has been recently demonstrated that acetic acid acts as an elicitor exerting itself a chitosan-like effect on xanthone biosynthesis (Valletta et al. 2016).

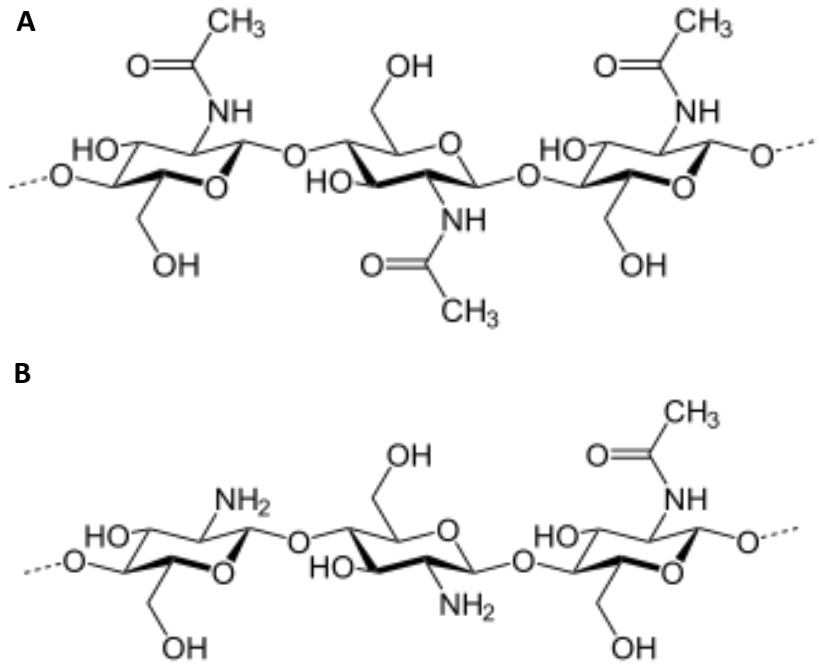

Fig. 10 Chemical structure of chitin (A) and chitosan (B).

1.7.1.2 Chitosan oligosaccharides

Chitosan oligosaccharides (COS) are obtained through chemical or enzymatic hydrolysis from chitosan and their use has recently increased. Unlike chitosan, COS are soluble in aqueous solutions in all proportions, due to the short chain length and to the free amino groups in D-glucosamine units (Jeon et al. 2000). Moreover, they are biodegradable, biocompatible, atoxic and have a low viscosity. These characteristics attracted researchers' attention for being promising oligosaccharides that have potentials in agriculture and in cosmetic, pharmaceutical and food industry. Several biological activities of COS have been demonstrated in recent years, including antimicrobial (Jeon and Kim 2000 and literature cited therein; Jeon et al. 2001; Choi et al. 2001), antitumoral (Nam et al. 1999; Jeon and Kim 2002; Nam et al. 2007; Shen et al. 2009), antioxidant (Xing et al. 2005), hypocholesterolemic (Kim et al. 2005), hypoglycemic (Miura et al. 1995), anti-Alzheimer's (Yoon et al. 2009) and accelerating calcium absorption (Jung et al. 2006). Moreover, COS have proven to promote plant growth, improving the capacity of plants against salt and drought stress (Dzung et al. 2011; Chatelain et al. 2014; Zou et al. 2015) and to be effective elicitors of innate immunity against diseases of plants such as tobacco, rice, grape and other (Agrawal et al. 2002; Eikemo et al. 2003; Cabrera et al. 2006; Chen et al. 2009). Studies on their use as biopesticides have been conducted, indeed they have a powerful protective effect on various species of plants of economic interest (Yin et al. 2010; Zhao et al 2007). They are an effective post-harvest treatment for inhibiting diseases, which affect many fruits such as citrus fruits, tomato, pear, apple and peach (Chien et al. 2007; Badawy and Rebea 2009; Meng et al 2010; Yang et al 2010, 2012; Yan et al. 2011). It was also demonstrated that a pre-harvest administration of COS determines a higher post-harvest resistance to pathogens (Yan et al. 2012; Ma et al. 2013). Their biological activity strictly depends on its chemical and physical properties such as viscosity, polymerization degree and deacetylation degree (Cabrera et al. 2006 and literature cited therein; Zou et al. 2015).

1.7.1.3 Methyl jasmonate

Methyl jasmonate (MeJA) is a volatile compound which plays a long-distance signaling role in many cellular responses such as plant-environment, plant-herbivore and plant-plant interactions. In addition, MeJA and its precursor jasmonic acid (JA) are involved in many

developmental processes such as seed germination, root growth, stomatal closure, fruit maturation, leaf movement, leaf senescence, tuber formation and trichome formation (Creelman and Mulpuri 2002; Wasternack and Hause 2002; Wasternack 2014) (Fig. 11). Jasmonates (JA, MeJA and derivatives) are also involved in defense responses to biotic stress such as injury, wound caused by pests (Browse and Howe 2008), pathogen attack (Glazebrook 2005) and abiotic stress such as high salinity (Qiu et al. 2014), drought (Savchenko et al. 2014), cold (Du et al. 2013; Hu et al. 2013) and heat (Clarke et al. 2009). In stressed plants MeJA enhances protease inhibitors production against herbivores; these inhibitors cause the sensing of bad taste in pests and in some cases even cannibalistic tendencies. MeJA is also responsible for the production of phytoalexins, which act as antimicrobial agents. Moreover, a MeJA release to the atmosphere through stomata is recognized by nearby plants which activate defense responses (Farmer et al. 1990; Karban et al. 2000; Baldwin et al. 2006).

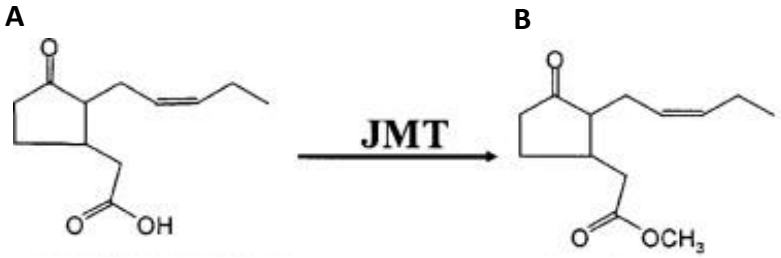

Fig. 10 Chemical structure of A) jasmonic acid and B) methyl jasmonate. JMT: JA carboxyl methyltransferase (Yang et al. 2006).

In plants MeJA is synthesized through the octadecanoid pathway (Fig. 11): external stimuli via the release of systemin cleaved from the C-terminal region of prosystemin, activate a phospholipase which releases α-linoleic acid from lipids of the chloroplast membrane. α-linoleic acid is oxygenated by specific lipoxygenases (LOX) to 13-hydroperoxylinoleic acid (13-HPOT), which is converted in 12,13-epoxyoctadecatrienoic acid by allene oxide synthase (AOS). On the latter compound acts allene oxide cyclase (AOC) to form 12-oxo-phytodienoic acid. This intermediate is then processed in peroxisomes through one reduction made by 12-oxo-phytodienoic acid reductase (OPR) and three β-oxidation cycles which form JA. The conjugation with isoleucine is required for its activation (JA-Ile). Free-acid JA might not be able to move across the cellular membrane because of its acidic characteristics so JA is catabolized by JA carboxyl methyltransferase (JMT) to MeJA (Yang et al. 2006; Browse et al. 2009 and literature cited therein) (Fig. 10).

Because of its role as signal molecule involved in defense responses, MeJA has been studied as elicitor in *in vitro* cultures of various plant species such as *Vitis vinifera* (Repka et al. 2004), *Rubus* sp. (Wang et al. 2008), *Mentha piperita* (Krzyzanowska et al. 2012) and *H. perforatum* (Wang et al. 2015).

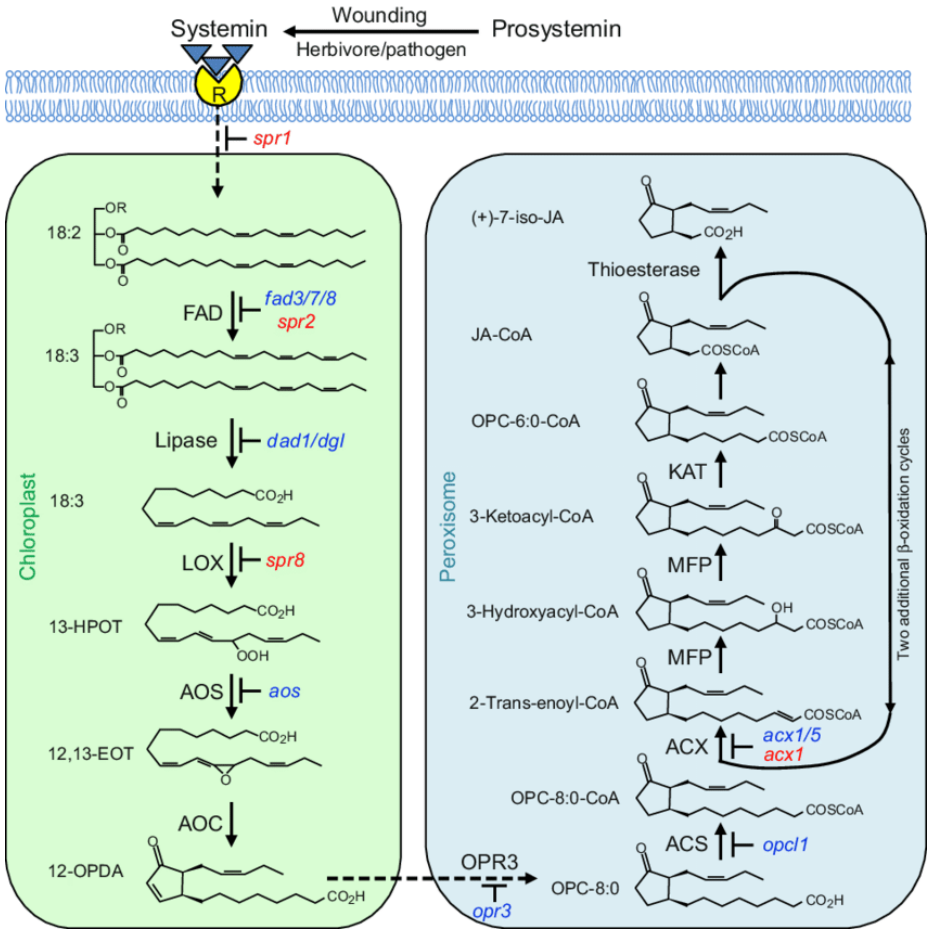

Fig. 11 Octadecanoid pathway for jasmonic acid (JA) biosynthesis (Zhai et al. 2017).

1.7.1.4 Salicylic acid

For two centuries, salicylic acid (SA) has been studied for its medicinal use in humans. Contrarily, its regulatory functions in plants as phytohormone have only been investigated in the last 30 years.

SA influences various processes in plant development and growth such as seed germination, cell growth, respiration, stomatal closure, senescence-associated gene expression, responses to abiotic stresses, and basal thermotolerance (Rate et al. 1999; Morris et al. 2000; Metwally et al. 2003; Clarke et al. 2004; Norman et al. 2004; Rajou et al. 2006; Clarke et al. 2009; and

literature cited therein). SA in plants is produced via two distinct pathways that require chorismate. This primary metabolite can be converted into SA via L-phenylalanine, involving a series of enzymatic reactions initially catalyzed by phenylalanine ammonia lyase (PAL) (Verberne et al. 1999). Chorismate can also be converted into SA via isochorismate in a two-step process involving isochorismate synthase (ICS) and isochorismate pyruvate lyase (IPL), well known in bacteria, but whose existence in plants is supported by various papers (Verberne et al. 2000; Wildermuth et al. 2001; Strawn et al. 2007). Genes regulated by SA can be divided into two classes: early-responsive genes, induced within 30 minutes of SA treatment, and genes induced later, including NPR1, a master regulator of the SA-mediated induction of PR genes. Most of the SA produced *in planta* is converted into SA O-β-glucoside (SAG) by a pathogen-inducible SA glucosyltransferase (SAGT). MeSA and/or its glucosylated derivative MeSAG also accumulates to relatively high levels *in vivo*. MeSA, obtained from SA by salicylic acid methyl transferase (SAMT) (Fig. 12), is probably involved in signal translocation to distal portions of the plant and then it is hydrolyzed by MeSA methyl esterase (SAPB2) to SA, activating the defense genes (Park et al 2007).
Treatments with exogenous SA have been investigated in *in vitro* cultures of diverse plant species enhancing plant resistance against both biotic and abiotic stress (Németh et al. 2002; Ali et al. 2006; Hussain et al. 2008; Popova et al. 2009; Sivanandhan et al. 2012; Gadzovska et al. 2013) making SA a cheap elicitor of plant defense responses.

Fig. 12 Chemical structure of A) salicylic acid and B) methyl salicylate. SAMT: salicylic acid methyl transferase (Yang et al. 2006).

1.7.1.5 Hydrogen peroxide

Hydrogen peroxide (H_2O_2) is a ROS whose production can be either enzymatic or non-enzymatic. There are numerous ways of H_2O_2 production in plant cells, such as photorespiration, electron transport chains (ETC), and redox reactions (Mittler 2002); in case

of high rates of production it is normally balanced by very efficient antioxidant systems which consist of both non-enzymatic and enzymatic. H_2O_2 scavengers include the enzymes catalase (CAT) (Willekens et al. 1997), peroxidase (POX) (Fan and Huang 2012), ascorbate peroxidase (APX) and glutathione reductase (GR) (Jahan and Anis 2014) (Fig. 13).

H_2O_2 has been considered mainly as a toxic cellular metabolite for decades; however, it is now clear that it acts also as a signaling molecule which may move between cells through aquaporin channels. H_2O_2 plays important roles in plant developmental and physiological processes including seed germination (Barba-Espín et al. 2011), PCD (Cheng et al. 2015; Vavilala et al. 2015), senescence (Liao et al. 2012), flowering (Liu et al. 2013), root system development (Liao et al. 2009; Ma et al. 2014; Hernández-Barrera et al. 2015), stomatal aperture regulation (Ge et al. 2015). This suggests that H_2O_2 is involved in cellular signaling transduction pathways and gene expression modulations in plants.

H_2O_2 is produced also through several enzymes including cell wall peroxidases (Francoz et al. 2015), oxalate oxidase (Hu et al. 2003), amine oxidases and flavin-containing enzymes (Cona et al. 2006). Moreover, nicotinamide adenine dinucleotide phosphate (NADPH) oxidases on the plasma membrane also increase H_2O_2 level through generating O_2^-, which in turn is converted to H_2O_2 and OH• by superoxide dismutases (SOD) (Grivennikova and Vinogradov 2013; Brewer et al. 2015).

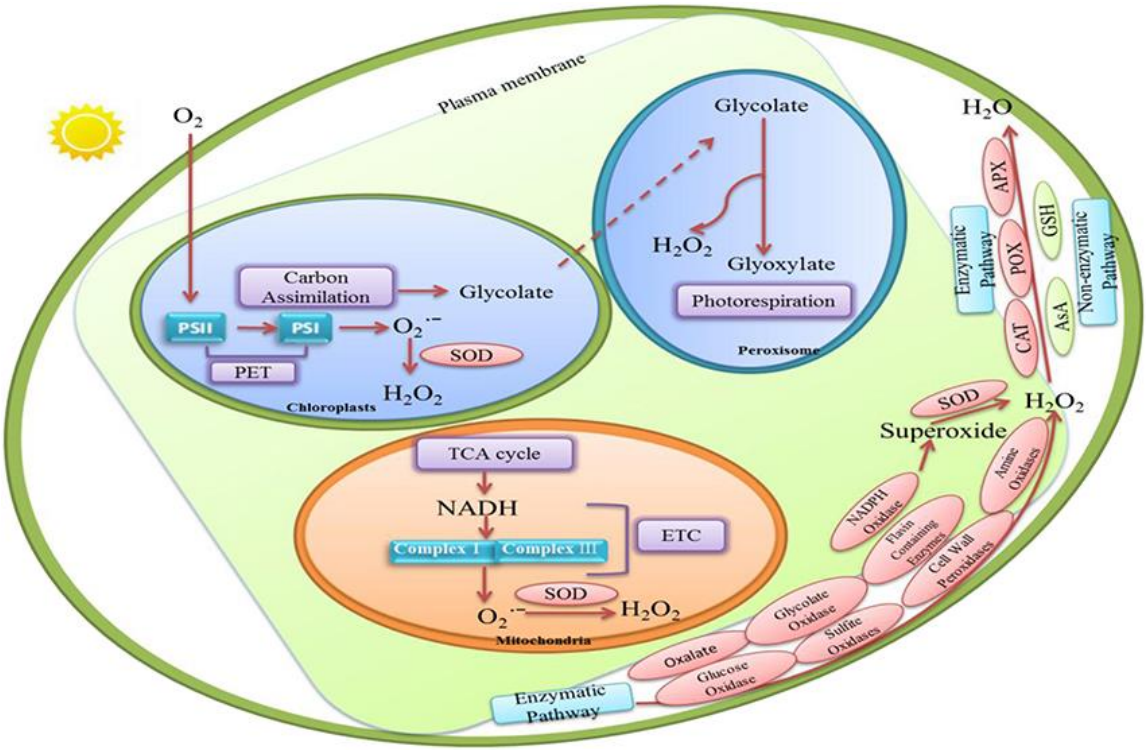

Fig. 13 Hydrogen peroxide (H_2O_2) production and removal in plant cells (Niu and Liao 2016).

An oxidative burst with rapid H_2O_2 synthesis and its release into the apoplast, is induced by biotic stress such as pathogens, elicitors and wounding or abiotic stress including drought (Ashraf et al. 2015), low and high temperatures (Orabi et al. 2015; Wu et al. 2015), salinity (Mohamed et al. 2015), ultra-violet light (He et al. 2013), ozone (Oksanen et al. 2004) and heavy metals (Wen et al. 2013), causing rapid responses in plant cells.

1.7.2 Abiotic stress

Abiotic stress is stress caused by non-living factors such as drought (Chaves and Oliveira 2004), salinity (Sahi et al. 2006; Munns and Tester 2008), heat (Scharf et al. 2012), cold (Chinnusamy et al. 2007), freezing (Sakai and Larcher 2012), nutrient (Hirel et al. 2007), high light intensity (Rossel et al. 2002), ozone (O_3) (Welfare et al. 2002) and anaerobic stresses (Agarwal and Grover 2006) (Wang et al. 2003; Nakashima et al. 2014). Abiotic stresses represent the primary cause of crop loss worldwide with reductions in production of more than 50% (Alcázar et al. 2006; Hussain et al. 2011) and probably these stresses will become more intense because of the expected climate change. Abiotic stresses can be used as plant defense stimulators to produce molecules of interest or to investigate plant responses pathways.

1.7.2.1 Toxic metals

Pollution with toxic metals is a phenomenon that is constantly increasing since it is closely connected to the anthropization process and to the exponential increase of human population. Alarming data show that in China 2.9% of the soils for agricultural use (corresponding to about 4 million hectares) is heavily contaminated by the presence of heavy metals (Su 2014), while in Europe 58% are characterized by quantities of toxic metals above the threshold values recommended and applied by UNEP (Tòth et al. 2016). The presence of toxic metals in the soil and their absorption by plants, can give rise to phenomena of food chain contamination, with repercussions either on animal and human health.

Heavy metal phytotoxicity may alter numerous physiological processes such as enzyme activity, metabolism of essential elements (Dong et al. 2006), and membrane integrity

(Gadallah 1999). Moreover, heavy metals enhance the production of reactive oxygen species (ROS) leading to oxidative stress.

Plants activate several defense mechanisms which control uptake, accumulation and translocation of heavy metals and detoxify them; furthermore, antioxidant systems which counteracts oxidative stress are activated (Srivastava et al. 2004). Although these plant mechanisms, heavy metals are often found in shoots, leaves, flowers, or, worse, seeds and fruits (Muchuweti et al. 2006; Unterbrunner et al. 2007; Shaheen et al. 2016). One common strategy is preventing the entrance of heavy metals into root cells by trapping them in the apoplast by detoxifying them via chelate complex formation (Watanabe and Osaki 2002) or to anionic groups of cell walls (Dalla Vecchia et al. 2005; Rascio et al. 2008). Most of the heavy metal amount that enters the plant is then kept in root cells, where it is detoxified by complexation with amino acids, organic acids or metal-binding peptides (e.g. phytochelatins) and/or sequestered into vacuoles (Salt and Rauser 1995; Piechalak et al. 2002). These trapping strategies protect the leaf tissues from damage.

The effects of high concentrations of heavy metals on plants are various and different depending on the pollutant, they include the reduction in photosynthesis, water and nutrient uptake, chlorosis, growth inhibition, browning of root tips, and death (Yadav 2010 and literature cited therein). Roots, being in direct contact with soils, are the most and first affected organ which show alterations both in their normal hormonal metabolism and in the development and morpho-anatomical differentiation, with damage that affects the growth of the entire plant.

In *Arabidopsis thaliana* and *Oryza sativa* it has been shown that both cadmium (Cd) and arsenic (As), respectively metal and half-metal toxic elements, frequently present in polluted soils, express their toxicity by altering both biosynthesis and transport of auxins, fundamental phytohormones for plant organogenesis (Ronzan et al. 2018; Fattorini et al. 2017). The correct distribution, carried out both through transport and conversion of the specific indole-3-butyric acid precursor (IBA) into its chemically active form indol-3-acetic acid (IAA), is required in various processes such as the genesis, development and maintenance over time of a functional root system (Strader et al. 2010). Moreover, effects of Cd on secondary metabolism were demonstrated in several species such as *Catharanthus roseus* (Zheng and Wu 2004), *Phyllanthus amarus* (Rai et al. 2005), *Brassica juncea* (Ahmad et al. 2016); otherwise, little is known about As effect on secondary metabolism.

Many species that survive in soils characterized by high heavy metal concentrations behave as “excluders”, they retain and detoxify most of the heavy metals in the root tissues, with a minimized translocation to the leaves (Hall 2002). Otherwise, the term “hyperaccumulator”

is used for plants which actively accumulate large amounts of one or more heavy metals from the soil and which translocate and accumulate them in aerial organs at concentrations hundreds-fold higher than non-hyperaccumulating species. These plants show no symptoms of phytotoxicity (Reeves 2006 and literature cited therein), and due to this, they could be more dangerous for human health especially in case of crops and medicinal plants. Among hyperaccumulating plants, there are species of numerous families such as Brassicaceae, Poaceae, Asteraceae, Fabaceae (Reeves et al 2006 and literature cited therein) and Hypericaceae including *H. perforatum* (Pavlova et al. 2015).

1.8 Research objectives

This work aims to elucidate the effect of biotic and abiotic stress on *H. perforatum* roots, administering chitosan oligosaccharides (COS), methyl jasmonate (MeJA), salicylic acid (SA), hydrogen peroxide (H_2O_2), cadmium (Cd) and arsenic (As). This project has both applicative and basic purposes: biotic elicitors may be used in order to stimulate secondary bioactive metabolite biosynthesis for drug production and to elucidate the influence of shoot/root interaction on elicitor perception in *H. perforatum*. Moreover, the treatment with toxic metals could help in understanding the processes that occurs when *H. perforatum* grows on polluted soils.

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Abbreviations chapter 2

CHIT	chitosan
COS	chitosan oligosaccharides
DVB/ CAR/PDMS	divinylbenzene/carboxen/polydimethylsiloxane
DW	dry weight
FDA	fluorescein diacetate
FW	fresh weight
GC-MS	chromatograph mass spectrometer
GI	growth index
HPLC	high performance liquid chromatography
IBA	indole butyric acid
SPME	solidphase- micro-extraction
VIP	variable importance in the projection
VOCs	volatile organic compounds

2. Chitosan oligosaccharides affect xanthone and VOC biosynthesis in *Hypericum perforatum* root cultures and enhance the antifungal activity of root extracts

Camilla Badiali[1] · Giulia De Angelis[1] · Giovanna Simonetti[2] · Elisa Brasili[1,3] · Eric de Castro Tobaruela[3] · Eduardo Purgatto[3] · Heng Yin[4] · Alessio Valletta[1] · Gabriella Pasqua[1]

[1] Department of Environmental Biology, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy

[2] Department of Public Health and Infectious Diseases, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy

[3] Department of Food Sciences and Experimental Nutrition/FORC-Food Research Center, University of São Paulo, Av. Prof. Lineu Prestes 580, São Paulo 05508-000, Brazil

[4] Liaoning Provincial Key Laboratory of Carbohydrates, Dalian Institute of Chemical Physics, Chinese Academy of Sciences, Dalian 116023, People's Republic of China

C. Badiali and G. De Angelis equally contributed to this work.

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Abstract

Key message Water-soluble chitosan oligosaccharides (COS) affect xanthone and volatile organic compound content, as well as antifungal activity against human pathogenic fungi of extracts obtained from *Hypericum perforatum* root cultures.

Several studies have demonstrated the elicitor power of chitosan on xanthone biosynthesis in root cultures of *H. perforatum*. One of the major limitations to the use of chitosan, both for basic and applied research, is the need to use acidified water for solubilization. To overcome this problem, the elicitor effect of water-soluble COS on the biosynthesis of both xanthones and volatile organic compounds (VOCs) was evaluated in the present study. The analysis of xanthones and VOCs was performed by HPLC and GC-MS headspace analysis. The obtained results showed that COS are very effective in enhancing xanthone biosynthesis. With 400 mg L^{-1} COS, a xanthone content of about 30 mg g^{-1} DW was obtained. The

antifungal activity of extracts obtained with 400 mg L^{-1} COS was the highest, with MIC_{50} of 32 μg mL^{-1} against *Candida albicans* and 32-64 μg mL^{-1} against dermatophytes, depending on the microorganism. Histochemical investigations suggested the accumulation of isoprenoids in the secretory ducts of *H. perforatum* roots. The presence of monoterpenes and sesquiterpenes was confirmed by the headspace analysis. Other volatile hydrocarbons have been identified. The biosynthesis of most VOCs showed significant changes in response to COS, suggesting their involvement in plant-fungus interactions.

Keywords *Hypericum perforatum* · Root cultures · Chitooligosaccharides · Xanthones · Volatile organic compounds

Introduction

Hypericum perforatum L. (Hypericaceae), popularly known as St. John's wort, has been one of the most investigated medicinal plants during the past two decades (Wölfle et al. 2014). The interest of scientific community towards *H. perforatum* mainly resides in its antidepressant activity (Russo et al. 2014), although it is being studied for a broad range of other biological activities (Marrelli et al. 2016).

Research on St. John's wort has focused primarily on metabolites accumulated in the aerial part of the plant, such as hypericins (naphthodianthrones) and hyperforins (phloroglucinols), that are believed to be responsible for the antidepressant activity (Russo et al. 2014). A large number of volatile organic compounds (VOCs) such as monoterpenes and sesquiterpenes with antibacterial and antifungal activities have also been detected in the shoot organs of wild plants and in *in vitro* shoot cultures of *H. perforatum* (Schwob et al. 2004; Pintore et al. 2005; Maggi et al. 2010; Guedes 2009). The root has not been recognized as a valuable source of bioactive compounds of pharmacological interest until last years, when several studies revealed the presence of bioactive polyphenols in the root of the plant and in *in vitro* regenerated roots (Bertoli et al. 2008; Cui et al. 2010a, b, c, 2011; Tocci et al. 2011, 2012, 2013a, b; Tusevski et al. 2013; Brasili et al. 2014; Zubrická et al. 2015; Simonetti et al. 2016; Valletta et al. 2016).

We have demonstrated that *in vitro* root cultures of St. John's wort produce xanthones at higher levels than the root of the plant (Tocci et al. 2011, 2012, 2013a; Simonetti et al. 2016; Valletta et al. 2016). Xanthones are a large and diverse group of non-flavonoid polyphenols produced by certain plants, fungi, lichens and bacteria (El-Seedi et al. 2009; Masters and Bräse 2012). These metabolites arouse great interest in the research community because of

their numerous pharmacological properties (for a review, see Negi et al. 2013). Xanthone rich extracts obtained from *H. perforatum in vitro* root cultures exhibited antifungal activity against several common human pathogenic fungi, such as *Candida* spp., *Cryptococcus neoformans*, dermatophytes and *Malassezia furfur* (Tocci et al. 2011, 2012, 2013a; Zubrická et al. 2015; Simonetti et al. 2016). In a recent study it has been demonstrated that root endodermis and exodermis are the cellular sites of xanthone biosynthesis in *H. perforatum in vitro* cultured roots (Tocci et al. 2018).

Chitosan (CHIT) is a natural non-toxic biopolymer, composed of randomly distributed β-(1→ 4)-linked d-glucosamine (deacetylated unit) and N-acetyl-d-glucosamine (acetylated unit), produced by the partial deacetylation of chitin, a major component of arthropod exoskeleton and fungal cell wall. Among elicitors, CHIT is one of the most commonly used to increase the biosynthesis of plant secondary metabolites of pharmacological interest. Several studies have shown the high effectiveness of CHIT in enhancing xanthone production in *H. perforatum* root cultures (Tocci et al. 2011, 2012, 2013a; Brasili et al. 2014; Simonetti et al. 2016); however, no studies on the impact of this elicitor on the production of VOCs are currently available.

Chitosan is poorly soluble in neutral water as well as in most organic solvents; therefore, it is commonly dissolved in water acidified with acetic acid, which greatly limits its application (Kim and Rajapakse 2005). In this regard, we have recently found that short-chain monocarboxylic acids, as acetic acid, could alter the xanthone profile, masking the effect of CHIT (Valletta et al. 2016). This problem could be overcome using water-soluble CHIT derivatives, also known as chitosan oligosaccharides or chitooligosaccharides (COS), which can be obtained by enzymatic and/or chemical hydrolysis of CHIT (Kim and Rajapakse 2005; Yin et al. 2010).

COS have been commercialized as low-calorie bulking agents since the 1980s and, more recently, they have gained interest in different fields, including food, agriculture, and medicine-related industries. As regards their application in the agri-food sector, most of the available studies focus on the administration of COS *in vivo*, in the field or in post-harvest, to fight microorganisms responsible for plant diseases, as well as for biodeterioration and mycotoxin contamination of food (Yin et al. 2010 and literature cited therein).

To date, only a few studies are available on the use of COS as elicitors to enhance the production of phytochemicals in plant *in vitro* cultures, and most of them have been performed on cell cultures (Cabrera et al. 2006; Wang et al. 2008). To the best of our knowledge, no studies have been published on *in vitro* root cultures.

The main objective of this study was to evaluate the elicitor power of COS on the biosynthesis of xanthones and VOCs in root cultures of *H. perforatum*. HPLC was used for the identification and quantification of six different xanthones, while GC-MS headspace analysis was adopted to determine the VOC profile. To compare the effect of COS with that of CHIT, COS were initially administered to the root cultures by following the same experimental design used in the previous studies for CHIT elicitation; subsequently, time- and concentration-dependent effects of COS on xanthone biosynthesis was investigated. The methanol extracts obtained from control and elicited roots were tested for their antifungal activity against human pathogens i.e. *Candida albicans*, *Trichophyton mentagrophytes*, and *Microsporum gypseum*.

Materials and methods

Plant material and root cultures

In vitro-regenerated roots of *H. perforatum* were obtained as previously described by Valletta et al. (2016). Liquid cultures were established as described by Valletta et al. (2016) with slight modifications. Briefly, 0.250 g fresh weight (FW) of roots was inoculated in 100 mL flasks containing 50 mL half-strength MS basal salts and vitamins (Murashige and Skoog 1962), supplemented with 1 mg L^{-1} IBA and 1.5% (w/v) sucrose. The MS medium and sucrose were purchased from Duchefa (Haarlem, The Netherlands), while the growth regulators were obtained from Sigma-Aldrich (Milan, Italy). The cultures were shaken at 100 rpm at 26 ± 1 °C and maintained in continuous darkness.

Chitosan oligosaccharides (COS) preparation and identification

Chitosan (molecular weight 300-500 kDa, minimum 95% deacetylated) was purchased from Jinan Haidebei Marine Bioengineering Co., Ltd. (Shandong, China). COS with a degree of polymerization (DP) of 2-10 were prepared through enzymatic hydrolysis of CHIT according to Zhang et al. (1999). In brief, chitosan was dissolved in 2% acetic acid. Enzyme mixture in 0.05 mol L^{-1} acetate buffer was added and the mixture was incubated for 30 min at 40 °C. The hydrolyzates were filtered on a hollow-fiber membrane. These crude COS were added to ethanol and the mixture was stirred, thus forming a supersaturated solution, and stored at 4 °C overnight. The insoluble precipitate was removed using filter paper. The received COS solution was vacuum dried to obtain COS powder. The degree of polymerization (DP) of the obtained COS was analyzed using hydrophilic interaction liquid

chromatography combined with CAD detector. The DP of the COS was from 2 to 10, the mean molecular weight was around 1 kDa, and the acetylation was less than 5%.

Elicitation

Elicitation with COS

The roots were elicited using COS dissolved in deionized water. Different COS concentrations (50-400 mg L^{-1}) were applied to the root cultures. The stock solutions were prepared with COS concentrations ranging from 10 to 80 g L^{-1} to add 250 µL to each flask. Elicitation was always carried out on day 8 of culture. The stock solutions were sterilized with a 0.2 µm syringe filter before being added to the liquid culture medium. Control samples were added with 250 µL deionized sterile water. Three different elicitation protocols were carried out, as described below and in Fig. 1.

Time-dependent xanthone biosynthesis. To investigate the xanthone biosynthesis in response to COS elicitation over time, the roots were elicited with 200 mg L^{-1} COS. Root samples were harvested by vacuum filtration on days 5, 10, 15, 20, and 25 post-elicitation, corresponding to days 13, 18, 23, 28, and 33 of culture (Fig. 1a).

Concentration-dependent xanthone biosynthesis. To determine the optimal elicitor concentration, the roots were elicited with 50, 100, 200, and 400 mg L^{-1} COS, and then harvested by vacuum filtration on day 25 post-elicitation, corresponding to day 33 of culture (Fig. 1b).

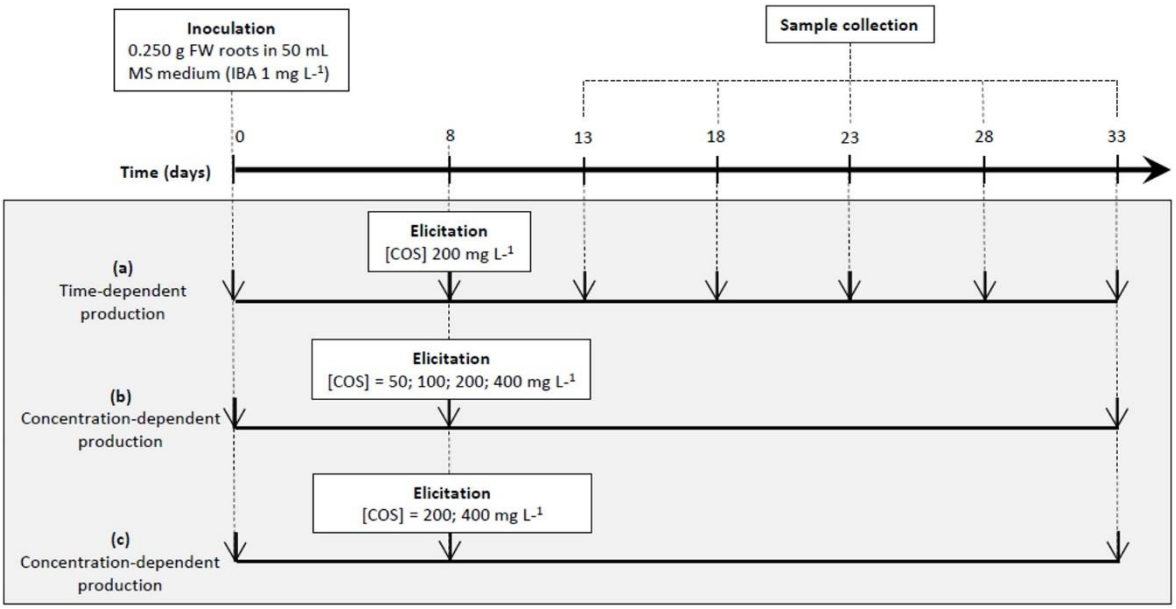


Fig. 1 Diagram representing protocols used to elicit *H. perforatum* root cultures

Concentration-dependent VOC biosynthesis. To investigate the effect of different COS concentrations on the volatile compounds profile, the roots were elicited with 200 and 400 mg L^{-1} COS. Root samples were harvested on day 15 after the elicitation (Fig. 1c).

Determination of root biomass

Growth curve of the *H. perforatum* roots, elicited with different COS concentrations (200 or 400 mg L^{-1}) and not elicited, was determined gravimetrically by measuring dry weight increases on days 5, 10, 15, 20, and 25 after the elicitation. The initial weight of all samples was 0.250 g FW of roots. The growth index (GI) was calculated as follows:

GI = Final weight – initial weight.

Xanthone quantification

The roots were dried in an oven at 70 °C until a constant weight was obtained; then, they were powdered with pestle and mortar and extracted three times (each 24 h) with methanol at room temperature. The ratio root dry biomass/methanol was 100/5 (mg:mL). The extracts were dried with a rotavapor (Buchi, Milan, Italy) at 35 °C and redissolved in HPLC-grade methanol (Carlo Erba, Milan, Italy) at the ratio initial biomass DW/methanol of 100/1 (mg:mL). The extracts were analyzed by high-performance liquid chromatography (HPLC), as described by Tocci et al. (2013a) and Valletta et al. (2016). Different xanthones were identified and quantified: (1) mangiferin; (2) 1,3,6,7-tetrahydroxyxanthone; (3) 1,3,5,6-

tetrahydroxyxanthone; (4) kielcorin; (5) cadensin G; (6) 1,7-dihydroxyxanthone; (7) toxiloxanthone, (8) paxanthone; and (9) 5-O-methyl-2-deprenylrheediaxanthone.

Histochemical detection of isoprenoids

Fresh sections (thickness ≈ 30 μm) of control roots and roots treated with 50, 100, 200, 400, and 800 mg L^{-1} COS and collected on day 18 of culture (corresponding to day 10 post-elicitation) (Fig. 1a), and were obtained by microtome (Vibratome Series 1000). The histochemical test with Nadi reagent was performed as previously reported by Monacelli et al. (2005).

Viability test

To assess the cytotoxicity of COS, a viability test with fluorescein diacetate (FDA) was carried out as previously reported by Santamaria et al. (2011) on *H. perforatum* cultured roots treated with 50, 100, 200, 400, and 800 mg L^{-1} COS on day 8 and collected on day 33 of culture. Non-treated roots were used as positive control and roots killed with liquid nitrogen were used as negative control. Roots were analyzed with a Zeiss microscope (Axioscop 2 Plus) fitted with a digital camera (Zeiss AxioCam MRc5) and a blue filter ($\lambda_{excitation}$ 386 nm; $\lambda_{emission}$ 490 nm). Roots that emitted a green fluorescence under blue light were considered viable.

Analysis of volatile organic compounds (VOCs)

The VOCs of roots treated with 200 or 400 mg L^{-1} COS and harvested on day 15 of culture were determined by solidphase- micro-extraction (SPME) that consists in catching the VOCs contained in the headspace above a sample in an SPME vial with a fiber coated with adapted stationary phases and inject them into a gas chromatograph mass spectrometer (GC-MS) with posterior data analyses.

SPME procedure

A divinylbenzene/carboxen/polydimethylsiloxane (DVB/ CAR/PDMS, 50 μm) fiber with manual holder from Supelco (Bellefonte, PA, USA) was used for the extraction of volatile compounds. The SPME fiber was preconditioned before the analyses, according to the instructions of the manufacturer.

The samples were ground using pestle and mortar under liquid nitrogen. A total of 3 g of roots was homogenized with 30% sodium chloride solution (Merck) and placed (10 g) into

a headspace vial sealed with a septum. The samples were kept under agitation with a magnetic stir bar and heated to 40 °C. The headspace equilibrium time was 30 min. Volatiles were extracted by exposing the SPME fiber to the headspace of the sample vial that was maintained at 40 °C for 60 min. For thermal adsorption, the SPME fiber was immediately inserted into the GC-MS injector and held for 2 min at 250 °C in splitless mode.

GC-MS conditions

Chromatographic analysis was performed in a Hewlett- Packard 6890 (Agilent Technologies Inc., Santa Clara, USA) GC-MS.

The injector temperature was 200 °C. Components were then separated using a capillary column Supelcowax 10 (30 m × 0.25 mm × 0.25 μm) and the oven temperature was programmed to ramp from 40 to 150 °C at 2 °C min^{-1} and hold for 5 min. Helium was used as a carrier gas with a constant column flow rate of 1 mL min^{-1}. The mass detector operated in electron impact (EI)-mode at 70 eV in a range of 15-210 amu. Volatile compounds were identified by comparison with the NIST database (NIST11, version 2.0, Gaithersburg, USA) and then confirmed with the Kovats retention indexes (RI). Further identification was carried out by calculating non-isothermal retention indices. The RI values were obtained by injecting saturated n-alkane standard solution (C7-C30 1,000 μg mL^{-1} in hexane, Supelco, Belgium) under the same chromatographic conditions and compared with those described in the literature determined under the same conditions for matching the compounds. The mass spectra data of all volatiles were also confirmed by comparison with the spectral data available at the MassBank of North America (MoNA—http://mona.fiehn lab.ucdav is.edu/).

Antifungal susceptibility testing

The evaluation of the antifungal activity was carried out on *C. albicans* ATCC 10231 coming from American-Type Culture Collection (ATCC, Manassas, VA, USA), *T. mentagrophytes* DSM 4870, and *M. gypseum* DSM 3824 coming from German Collection of Microorganisms (DSMZ, Braunschweig, Germany). To evaluate the minimal inhibitory concentration (MIC), the susceptibility *in vitro* assay was performed on *C. albicans* according to standardized methods for yeast using the broth microdilution method (CLSI M27-A3 2008b; CLSI 2012) and on dermatophytes according to standardized methods for filamentous fungi (CLSI M38-A2 2008a). Dermatophytes were grown on potato dextrose agar (Sigma-Aldrich, St. Louis, MO, USA) at 28-30 °C until good conidial growth was present. The conidia suspension was prepared at the final concentration of 1 × 103 to 3 × 103 CFU mL^{-1} (CLSI M38-A2 2008a). *C. albicans* was grown on Sabouraud dextrose agar at 35 °C for 24 h. The final concentration

of the inoculum was 0.5 × 103-2.5 × 103 CFU mL^{-1}. The *in vitro* antifungal susceptibility was evaluated using extracts. The concentration of extracts ranged from 512 to 0.5 μg mL^{-1}. The MIC_{50} was the lowest concentration of extracts or reference drugs that caused ≥ 50% growth inhibition and the MIC_{100} was the lowest concentration that inhibited 100% of growth. Results were expressed as median of three experiments performed in duplicate.

Statistical analysis

All measurements were made at least in triplicate and the results were expressed as means ± SD. Statistical analysis was carried out using SigmaPlot 13.0. Two-way analysis of variance (ANOVA), followed by Holm-Sidak tests, was applied to test differences between groups. Statistical significance of the results was also evaluated, also by paired Student's *t* test, and differences with a *p* value ≤ 0.05 were considered significant. A total of six biological replicates of samples for each treatment were analyzed by GC-MS. Volatile organic data set was imported into Metaboanalyst 3.0 (http://www.metaboanal yst.ca) for multivariate statistical analysis. All imported data were Pareto-scaled. A principal component analysis (PCA) was conducted on GC-MS data to discern inherent similarities in volatiles profiles. Next, a PLS-DA model was used to maximize covariance between the measured data (concentrations in GC-MS spectra, X matrix) and the response variable (predictive classifications, Y matrix). The variable importance in the projection (VIP) plot was then used to identify which volatile compound contributes most to clustering or trends observed in the data.

Results

Elicitation with COS

Root biomass growth

The increase in root biomass growth was monitored on days 0 and 8 of culture and every 5 days after elicitation with different COS concentrations (Fig. 2). During the first 8 days, a doubling of root biomass was observed in all analyzed roots. In control roots, the exponential growth phase began on day 8 and continued until day 18; a decrease in biomass growth was observed from day 18 to day 23 (non-significant differences); starting from day 23 to day 28, a plateau was recorded (non-significant differences); another exponential growth phase took place from day 28; and the elicitation on day 8 causes a remarkable decrease of growth in treated roots. The main difference between roots elicited with 200 and 400 mg L^{-1} took place at day 33, after which, the roots treated with the lower COS concentration slowly started to grow again.

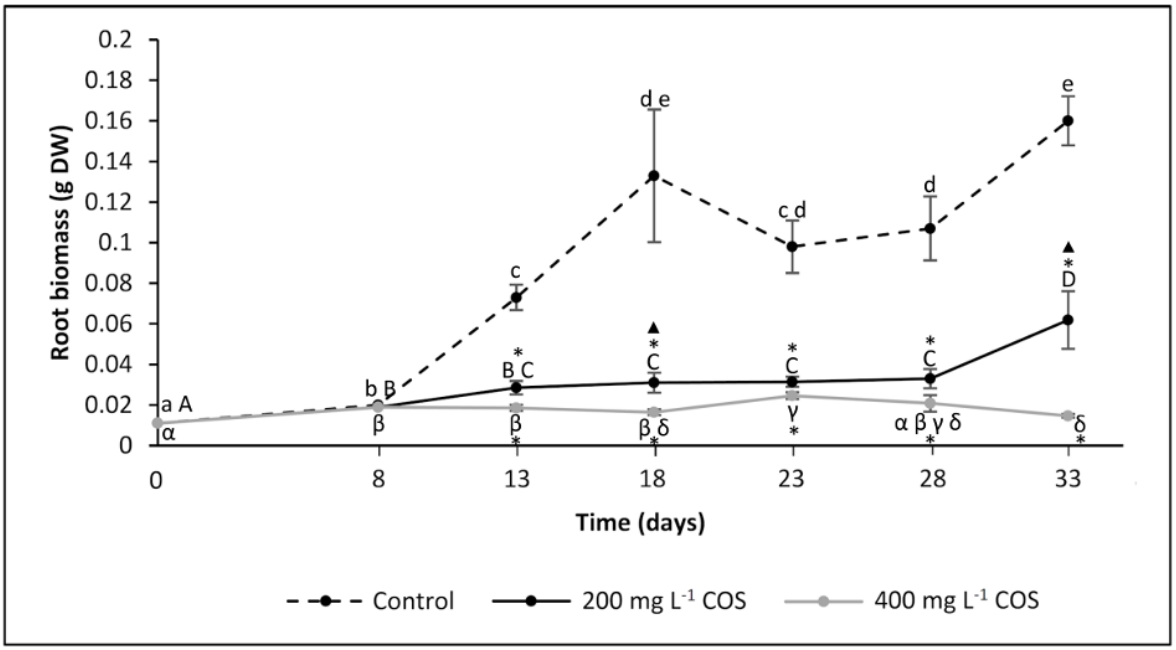


Fig. 2 Growth curves of *H. perforatum* root cultures elicited with 200 and 400 mg L^{-1} COS. Control roots (black dashed line); treated roots with 200 mg L^{-1} COS (black line); treated roots with 400 mg L^{-1} COS (grey line). Mean values were based on three replicates from two separate experiments. Bars represent standard deviations of the means. Different letters represent significant differences between samples ($p \leq 0.05$); asterisk represents significant differences between samples compared with control at the same day of culture; black up-pointing triangle represents significant differences between samples compared with 400 mg L^{-1} COS at the same day of culture

Time-dependent xanthone biosynthesis

The HPLC analysis showed that the addition of 200 mg L^{-1} COS to *H. perforatum* root cultures significantly enhances xanthone biosynthesis. All extracts obtained from COS-elicited roots and collected at different times post-elicitation (days 5, 10, 15, 20, and 25) (Fig. 1a) showed a significant increase in total xanthones compared to their respective controls (Fig. 3). In the control roots, total xanthone content increased with an increasing culture time from about 3.42 to 7.87 mg g^{-1} DW. In COS-elicited roots, the highest xanthone levels were observed after a short time (day 5) and after a long-time (day 25) post-elicitation, with 13.25 e 14.31 mg g^{-1} DW of total xanthones, respectively. On days 10, 15, and 20, xanthone content remained almost constant with an average value of about 9.8 mg g^{-1} DW.

The levels of individual xanthones were measured at all experimental times (supplementary material), and in Fig. 4, the data acquired at times of maximum production (days 5 and 25) are shown. On day 5 (Fig. 4a), all the analyzed xanthones were present in control roots, with the exception of kielcorin (Kiel) and 1,7-dihydroxyxanthone (Dihydroxy). Mangiferin (Mang) and toxyloxanthone (Toxy) were accumulated at relatively low levels (0.22-0.18 mg g^{-1} DW). Other xanthones were accumulated at levels ranging from 0.55 for paxanthone (Pax) to 1.02 mg g^{-1} DW for 5-O-methyl-2-deprenylrheediaxanthone (Rheedia). The levels of all xanthones significantly increased in response to COS elicitation, with the exception of mangiferin (Mang) and cadensin G (Cad). The highest increases were observed for tetrahydroxixanthones (Tetra), Toxy, Pax, and Rheedia (4.6-, 17.0-, 5.6-, and 2.8-fold increase, respectively). On day 25 (Fig. 4b), all analyzed xanthones were detected in control roots, with the exception of Kiel and Dihydroxy. Mang, Tetra, and Cad were accumulated at relatively low levels (0.31, 0.29, and 0.51 mg g^{-1} DW). Toxy, Pax, and Rheedia were accumulated at higher levels (1.02, 1.04, and 4.7 mg g^{-1} DW).

In response to COS, the levels of all analyzed xanthones increased, with the exception of the Mang, the level of which decreased significantly. These increases were statistically significant for Tetra, Cad, Pax, and Rheedia (3.8-, 0.22-, 4.2-, and 1.3-fold increase, respectively).

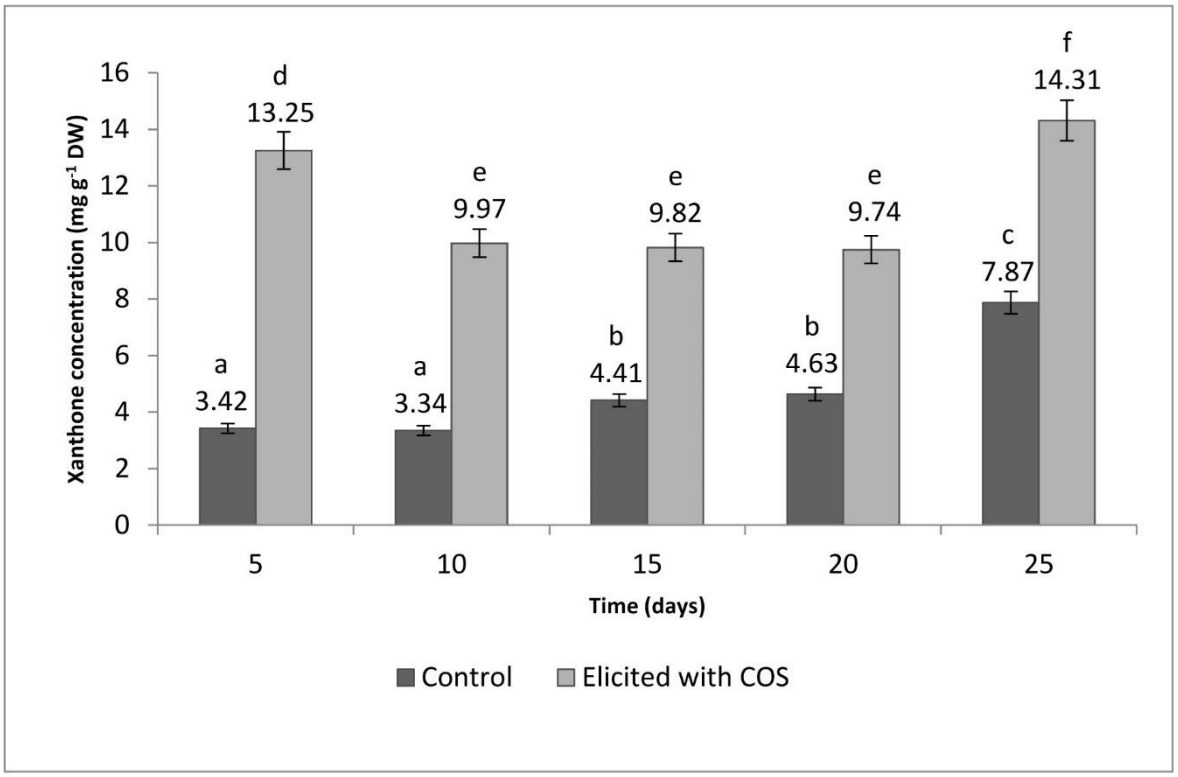
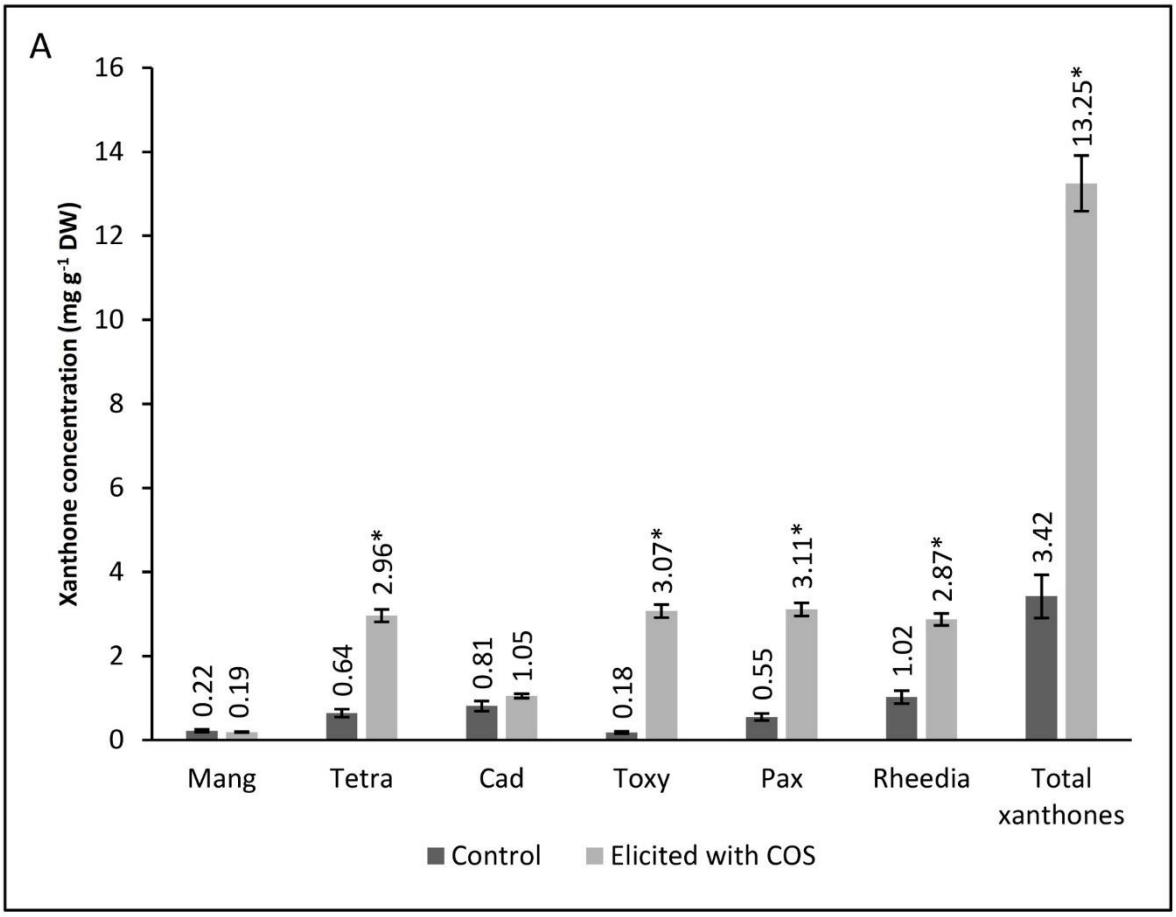


Fig. 3 Time-dependent xanthone production in *H. perforatum* root cultures in response to elicitation with 200 mg L^{-1} COS. The numbers next to the bars represent the exact concentration of total xanthones (mg g^{-1} DW). The data shown are mean of three replicates from two separate experiments. Bars represent standard deviations of the means. Different letters represent significant differences between samples ($p \leq 0.05$)


A
Xanthone concentration (mg g-1 DW)
16
14
12
10
8
6
4
2
0
0.22
0.19
0.64
2.96*
0.81
1.05
0.18
3.07*
0.55
3.11*
1.02
2.87*
3.42
13.25*
Mang
Tetra
Cad
Toxy
Pax
Rheedia
Total xanthones
Control
Elicited with COS

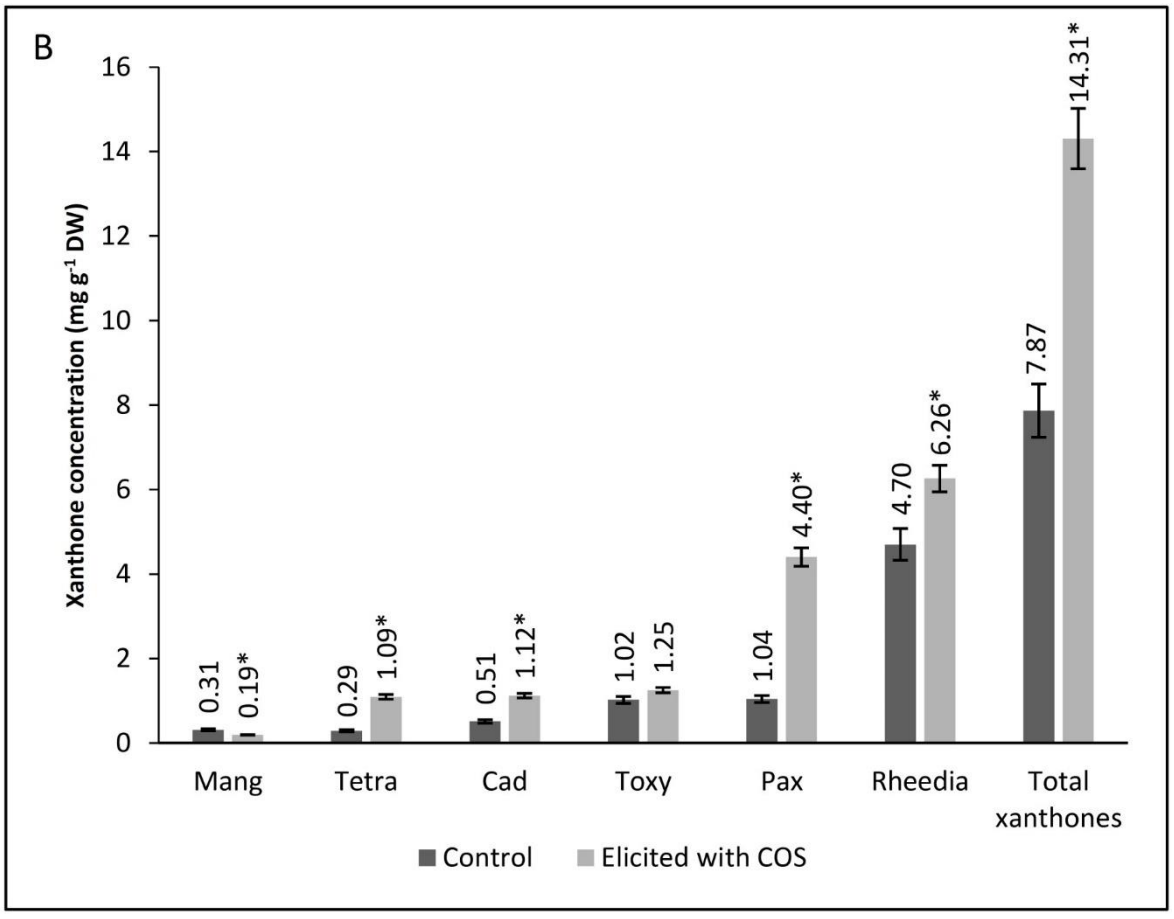

B
Xanthone concentration (mg g-1 DW)
16
14
12
10
8
6
4
2
0
0.31
0.19*
0.29
1.09*
0.51
1.12*
1.02
1.25
1.04
4.40*
4.70
6.26*
7.87
14.31*
Mang
Tetra
Cad
Toxy
Pax
Rheedia
Total xanthones
Control
Elicited with COS

Fig. 4 Xanthone production in *H. perforatum* root cultures on days 5 (a) and 25 (b) after elicitation with 200 mg L^{-1} COS. The numbers next to the bars represent the concentration of xanthones (mg g^{-1} DW). The data shown are mean of three replicates from two separate experiments. Bars represent standard deviations of the means. Asterisks indicate statistically significant differences ($p \leq 0.05$) between COS-elicited roots and control roots. Mang mangiferin, Tetra 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone, Cad cadensin G, Toxy toxyloxanthone, Pax paxanthone, Rheedia 5-O-methyl-2-deprenylrheediaxanthone

Concentration-dependent xanthone biosynthesis

COS at different concentrations were tested on *H. perforatum* root cultures. Chemical analyses were performed on roots collected on day 25 post-elicitation, which gave the best results in the previous experiments. The roots treated with 400 mg L^{-1} COS were the most productive in term of total xanthones (Fig. 5), the content of which was about 12 times higher than in control roots (30.8 and 2.5 mg g^{-1} DW, respectively). At higher concentrations, the xanthone content significantly decreased (data not shown) and the roots showed symptoms of necrosis (Fig. 8). In the roots elicited with 400 mg L^{-1} COS, all analyzed xanthones were produced at much higher levels than roots treated with lower COS concentrations, with the exception of Mang (Fig. 6).

The highest levels of Mang were detected in roots treated with 50 e 100 mg L^{-1} COS (1.95-1.71 mg g^{-1} DW). As regards Tetra, Cad, Pax, and Rheedia levels in elicited roots, it was 15.4-, 23.4-, 41.3-, and 15.4-fold higher than in control roots. Toxy was not detected in untreated roots, while it was produced at relatively high levels (7.06 mg g^{-1} DW) in roots treated with 400 mg L^{-1} COS.

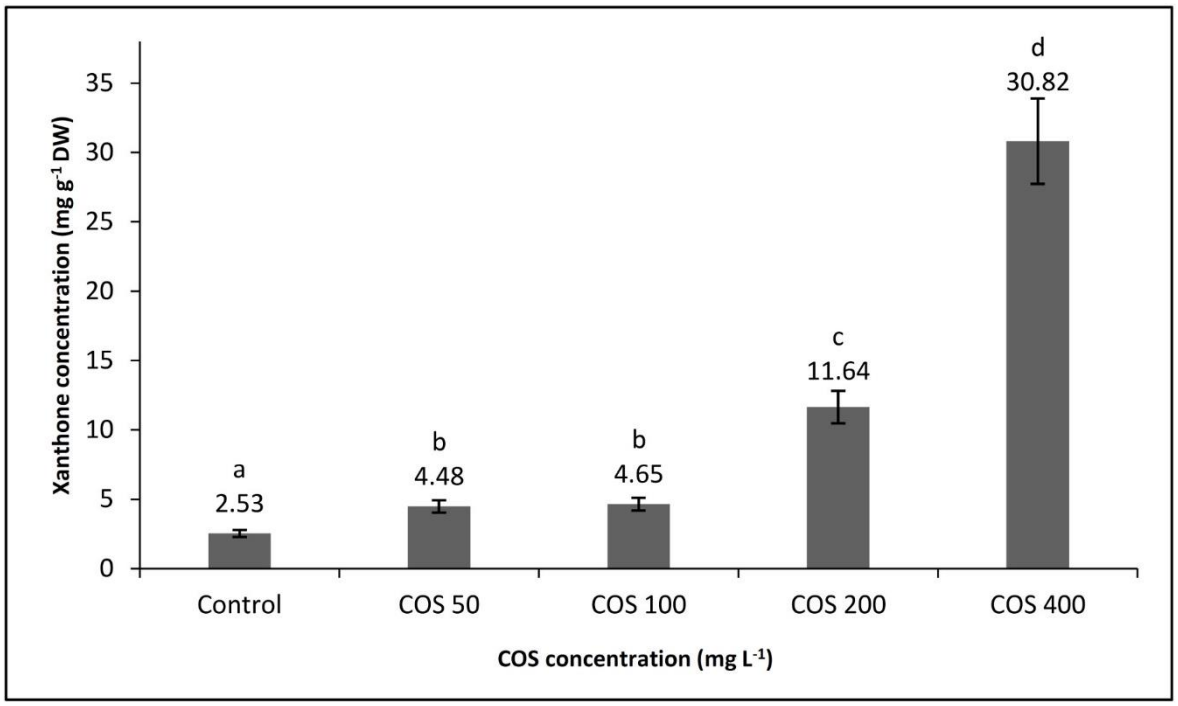


Fig. 5 Total xanthone content in *H. perforatum* root cultures not subjected to COS elicitation (control) or elicited with different COS concentrations. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Each value is the mean of three independent determinations ± SD. Different letters represent significant differences between samples ($p \leq 0.05$)

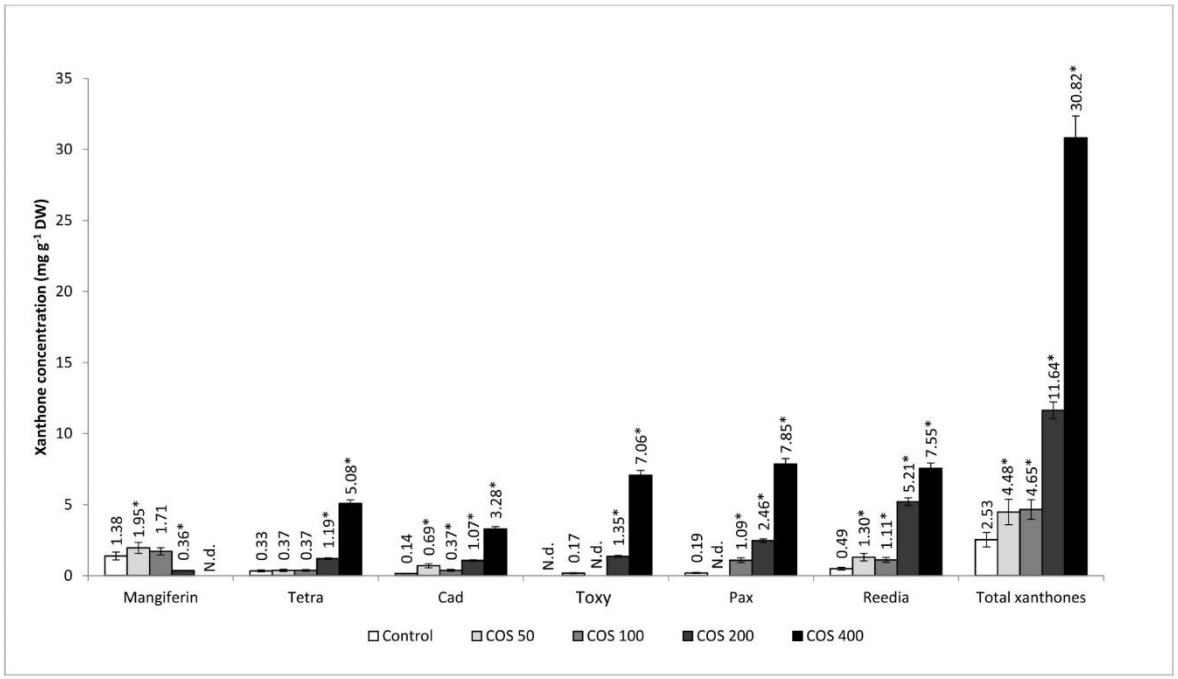


Fig. 6 Xanthone content in *H. perforatum* root cultures not subjected to COS elicitation (control) or elicited with different COS concentrations. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Each value is the mean of three independent determinations ± SD. Asterisks indicate that the differences between COS-elicited roots and the corresponding control roots are

statistically significant ($p < 0.05$). Mang mangiferin, Tetra 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone, Cad cadensin G, Toxy toxyloxanthone, Pax paxanthone, Rheedia 5-O-methyl-2-deprenylrheediaxanthone, N.d. non-detected

Histochemical detection of isoprenoids

The treatment with Nadi reagent revealed secretory ducts in both control and COS-treated roots, each delimited by four secretory cells (Fig. 7). Both the secretory cells and the lumen of the ducts reacted positively with Nadi reagent, which revealed the presence of isoprenoid compounds. As regards the signal intensity, no differences were observed between control roots and roots treated with different concentrations of COS (Fig. S1).

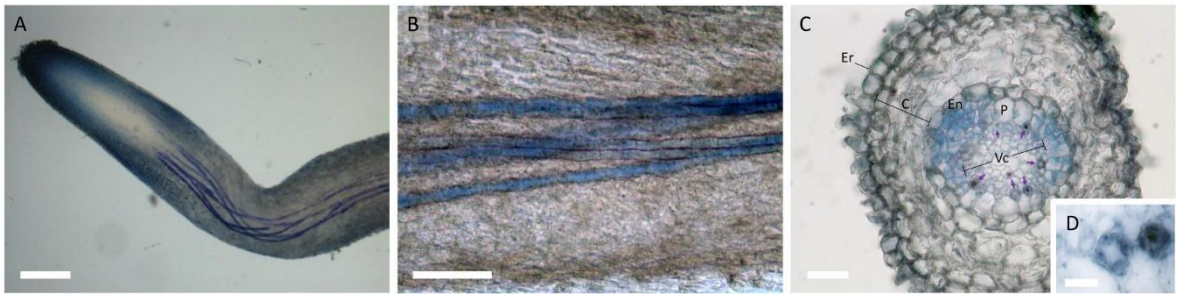


Fig. 7 *In vitro* roots of *H. perforatum* elicited with 200 mg L^{-1} COS, treated with Nadi reagent and observed under bright field. Intact root at different magnifications (A, B); root cross section (C); detail of the root section in which two close secretory ducts are visible (D). Er root epidermis, C cortex, En endodermis, P pericycle, Vc vascular cylinder. Bars represent 500 (A), 200 (B), 100 (C) and 25 μm (D)

Viability test

H. perforatum cultured roots subjected to COS concentrations ≤ 200 mg L^{-1} (Fig. 8A) showed a macroscopic appearance similar to non-treated roots (Fig. 8b, c). Symptoms of suffering in the form of tissue darkening and morphological alterations were observed in roots treated with COS concentrations > 200 mg L^{-1} (Fig. 8D-F). The viability of non-treated roots and roots elicited with COS concentrations ranging from 50 to 800 mg L^{-1} was investigated through FDA-viability test. The cells of non-treated roots and roots treated with 50-800 mg L^{-1} COS emitted a green fluorescence when observed with a microscope under blue light (Fig. 8H-M). No fluorescent signal has been observed in roots treated with liquid

nitrogen (Fig. 8N). These results indicate that COS does not cause cell death at the tested concentrations.

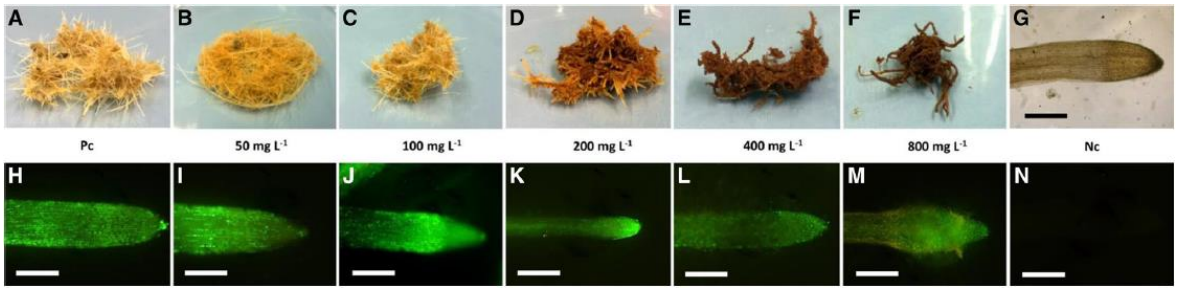


Fig. 8 Fluorescein diacetate (FDA) viability test on *H. perforatum* roots collected on day 33 of culture (corresponding to day 25 post-elicitation). Macroscopic appearance of non-treated roots (A) and roots subjected to different COS concentrations (B-F). Roots observed through epifluorescent microscopy under blue light to reveal the green signal generated by FDA (H-N). Positive control (Pc) represented by non-treated roots (A, H) and negative control (Nc) represented by roots killed with liquid nitrogen (G, N). Bars represent 300 μm

Antifungal activity of extracts obtained from *H. perforatum* cultured roots elicited with COS

The antifungal activity of extracts obtained from *H. perforatum* cultured roots elicited with different COS concentrations and collected on different days post-elicitation has been evaluated against *C. albicans, T. mentagrophytes* and *M. gypseum*. As regards MIC_{50} and MIC_{100} of *C. albicans* and MIC_{50} of *M. gypseum*, the best activity was exhibited by extracts of roots collected on days 20 and 25 post-elicitation (32, 64, and 16 μg mL^{-1}, respectively). As regards MIC_{100} of *M. gypseum*, the best activity was exhibited by extracts of roots collected on days 15 and 20 post-elicitation (32 μg mL^{-1}). Antifungal tests performed against *T. mentagrophytes* with extracts obtained from roots collected at different experimental times, yielded similar MIC values (MIC_{50} 16 μg mL^{-1}; MIC_{100} 32 μg mL^{-1}) (Tables 1 and S1).

Moreover, the antifungal activity increased by increasing the COS concentration (Tables 2 and S2). COS did not show any antifungal activity on the investigated fungal strains.

Table 1. Antifungal activity of methanol extracts of *H. perforatum* root cultures collected at different days after elicitation with 200 mg L^{-1} COS against *Candida albicans*, *Trichophyton mentagrophytes* and *Microsporum gypseum*.

Extracts	*C. albicans* ATCC 10231		*T. mentagrophytes* DSM 4870		*M. gypseum* DSM 3824	
	MIC_{50} (μg mL^{-1})	MIC_{100} (μg mL^{-1})	MIC_{50} (μg mL^{-1})	MIC_{100} (μg mL^{-1})	MIC_{50} (μg mL^{-1})	MIC_{100} (μg mL^{-1})
Control day 5	256	256	64	128	128	256
EL day 5	64 (*a)	256 (a)	16 (*a)	32 (*a)	32 (*a)	64 (*a)
EL day 10	64 (*a)	128 (*b)	16 (*a)	32 (*a)	32 (*a)	64 (*a)
EL day 15	32 (*b)	128 (*bc)	16 (*a)	32 (*a)	32 (*a)	32 (*b)
EL day 20	32 (*b)	64 (*c)	16 (*a)	32 (*a)	16 (*b)	32 (*b)
EL day 25	32 (*b)	64 (*cd)	16 (*a)	32 (*a)	16 (*b)	64 (*a)
Fluconazole	2 (*c)	64 (*d)	16 (*a)	32 (*a)	16 (*b)	32 (*b)

MIC_{50} and MIC_{100} are the lowest concentration of extracts or reference drugs that caused growth inhibition ≥ 50% and 100%, respectively. Results are expressed as median of three experiments performed in duplicate. Asterisks represent significant differences ($p \leq 0.05$) between MIC values obtained with extracts from COS-elicited roots (EL) and from non-treated roots (control). Different letters represent significant differences ($p \leq 0.05$) between MIC values reported in each column.

Table 2. Antifungal activity of methanol extracts of *H. perforatum* root cultures elicited with COS at different concentrations and collected at day 25 post-elicitation against *Candida albicans*, *Trichophyton mentagrophytes* and *Microsporum gypseum*.

Extracts	*C. albicans* ATCC 10231		*T. mentagrophtyes* DSM 4870		*M. gypseum* DSM 3824	
	MIC_{50} (μg mL^{-1})	MIC_{100} (μg mL^{-1})	MIC_{50} (μg mL^{-1})	MIC_{100} (μg mL^{-1})	MIC_{50} (μg mL^{-1})	MIC_{100} (μg mL^{-1})
Control	256	512	128	512	128	256
EL (50 mg L^{-1})	256 (a)	512 (a)	128 (a)	256 (*a)	128 (a)	256 (a)
EL (100 mg L^{-1})	64 (*b)	256 (*b)	64 (*b)	256 (*a)	64 (*b)	128 (*b)
EL (200 mg L^{-1})	32 (*c)	128 (*c)	64 (*b)	128 (*b)	64 (*b)	128 (*b)
EL (400 mg L^{-1})	32 (*c)	128 (*c)	64 (*b)	64 (*c)	32 (*b)	64 (*b)
Fluconazole	2 (*d)	64 (*d)	16 (*c)	32 (*d)	16 (*c)	32 (*c)

MIC_{50} and MIC_{100} are the lowest concentration of extracts or reference drugs that caused growth inhibition ≥ 50% and 100%, respectively. Results are expressed as median of three experiments

performed in duplicate. Asterisks represent significant differences ($p \leq 0.05$) between MIC values obtained with extracts from COS-elicited roots (EL) and from non-treated roots (control). Different letters represent significant differences ($p \leq 0.05$) between MIC values reported in each column.

Concentration-dependent volatile organic compounds (VOCs) biosynthesis

As shown in Table S3, VOCs were identified and listed according to their biosynthetic origin. A total of 43 volatile compounds were identified, including fatty acid derived volatiles, phenylpropanoid/benzenoid compounds, acyclic, monocyclic and bicyclic monoterpenes, and sesquiterpenes. The values of relative peak areas (median normalized) obtained by SPME-GC-MS for each compound constitute an estimate and do not reflect the actual value of volatile compounds in root samples, but are merely a parameter to compare the effect of different COS concentrations on the volatile profile of *H. perforatum* roots. Primarily, a PCA was applied to explore the volatile data set and to highlight the differences. The first two principal components (PC1 and PC2) explained 44.1% of total variability among the samples and showed a separation between root samples (Fig. S2). PC1 separated 200 mg L^{-1} COS-treated roots from 400 mg L^{-1} COS-treated and control roots due to their characteristic volatile profile. Interestingly, roots treated with 400 mg L^{-1} of COS and control roots were not distinguished by PC1.

Next, PLS-DA was applied to minimize the possible contribution of intergroup variability and to improve the separation between the samples. The PLS-DA score plot (R2Y = 0.89, Q2 = 0.80) showed a clear differentiation between control, the roots treated with COS 200 and 400 mg L^{-1} (Fig. 9).

According to the results obtained by PLS-DA, 15 volatile organic compounds were statistically significant as showed by VIP values (VIP > 1.0) in Table 3. Samples treated with COS 200 mg L^{-1} presented a characteristic volatile profile containing sesquiterpenes such as seychellene, cis-β- farnesene and (+)-α-chamigrene that were not identified in CTRL and COS 400 mg L^{-1} treated roots. Estimations of VOCs content in the roots indicated that only two sesquiterpenes (+)-δ-cadinene and (+)-epi-bicyclo-sesquiphellandrene increased after elicitation with the increasing COS concentration.

Interestingly, the majority of VOCs decreased or increased after elicitation with COS 200 mg L^{-1} and then returned to similar levels as CTRL roots. In particular, α-copaene, α-patchoulene, and cadina-3,5-diene increased after COS 200 mg L^{-1} and decreased after COS 400 mg L^{-1} treatment, while myrtenal, myrtanol, and limonene decreased after COS 200 mg L^{-1} and increased after COS 400 mg L^{-1} treatment. (−)-Zingiberene and 1,2,4-

trimethylbenzene were not detected after COS 200 mg L^{-1} treatment, but a decrease of them was observed after COS 400 mg L^{-1} compared to CTRL roots.

Conversely, naphthalene and α-terpineol were reduced after elicitation with COS 200 mg L^{-1}, but were not detected after elicitation with COS 400 mg L^{-1}.

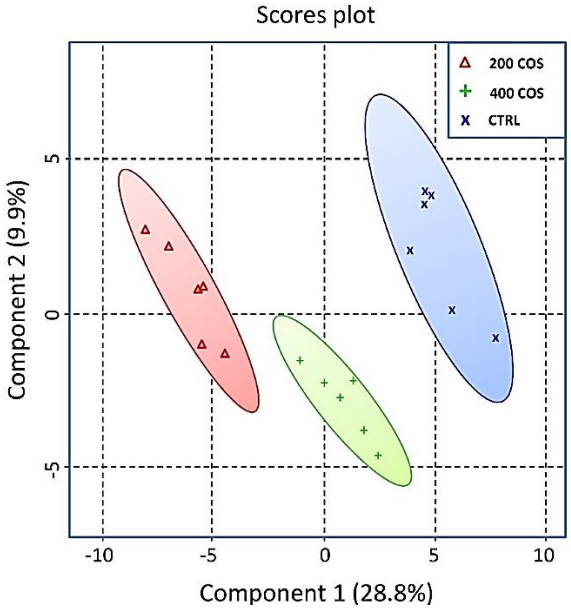


Fig. 9 PLS-DA score plot of the *H. perforatum* roots analyzed by SPME-GC-MS methodology. Control samples (CTRL) are represented in blue; COS-treated samples are represented in red (200 mg L^{-1}) and in green (400 mg L^{-1}).

Table 3 Volatile organic compounds statistically significant after COS treatment

Compounds	Control	200 mg L^{-1} COS	400 mg L^{-1} COS	VIP
Seychellene	-	18.29 ± 7.31	-	2.01
Naphthalene	3.16 ± 2.26	3.13 ± 1.94	-	1.99
(−)-Zingiberene	2.42 ± 0.62	-	1.53 ± 0.27	1.87
α-Terpineol	2.21 ± 1.69	0.42 ± 0.06	-	1.73
Cis-β-farnesene	-	2.87 ± 1.12	-	1.67
α-Copaene	8.70 ± 0.05	11.59 ± 3.65	9.37 ± 2.98	1.64
1,2,4-Trimethylbenzene	0.33 ± 0.19	-	0.21 ± 0.07	1.52
(+)-α-Chamigrene	-	0.45 ± 0.30	-	1.25
Myrtenal	4.31 ± 3.92	0.66 ± 0.21	0.97 ± 0.36	1.20
Limonene	0.46 ± 0.25	0.15 ± 0.02	0.38 ± 0.11	1.19
α-Patchoulene	2.77 ± 0.32	3.87 ± 1.12	2.11 ± 0.78	1.19
(+)-δ-Cadinene	0.54 ± 0.03	1.30 ± 0.51	2.06 ± 0.66	1.17
Myrtanol	6.64 ± 4.38	1.29 ± 0.26	4.64 ± 1.79	1.05
(+)-Epi-bicyclo-sesquiphellandrene	1.03 ± 0.09	1.51 ± 0.53	2.10 ± 2.22	1.00
Cadina-3,5-diene	1.29 ± 0.16	1.79 ± 0.59	1.34 ± 0.32	1.00

All the values are the mean of three independent analyses ± SD *VIP* variable importance in the projection

Discussion

Several studies have demonstrated the elicitor effect of CHIT on plant cells (Vasconsuelo et al. 2003; Fan et al. 2010) and organ cultures (Putalun et al. 2007; Sivanandhan et al. 2012). A limitation to the use of CHIT in *in vitro* cultures, as well as in field and in post-harvest, is the poor solubility in neutral water and organic solvents, which makes it necessary to use acidified water for solubilization. In most of the elicitation studies, CHIT is dissolved in acetic acid-water solutions. However, we recently demonstrated that monocarboxylic acids could affect the xanthone profile of *H. perforatum in vitro*-cultured roots and this makes it difficult to discriminate the effect of CHIT to that of solvent (Valletta et al. 2016). For this reason, we evaluated the effect of water-soluble derivatives of CHIT, named chitooligosaccharides (COS), obtained through enzymatic digestion of CHIT in this study. At present, only few studies are available on the impact of COS on secondary metabolite production in plant cell cultures (Linden and Phisalaphong 2000), and to the best of our knowledge, no studies have been performed on root cultures.

In this context, an issue we addressed in the present study was whether COS have an elicitor power comparable to that of CHIT on xanthone biosynthesis in *H. perforatum* root cultures. This is not a trivial query, since it is well known that the effect of CHITs on plant cells is strongly affected by their structural properties, e.g., molecular weight and degree of acetylation (Iriti and Faoro 2009 and literature therein reported). In a previous study we tested different CHITs on *H. perforatum* root cultures and we found significant differences in the elicitor power related to different molecular structures (Tocci et al. 2013a). To compare the effect of COS with those of CHIT, the first elicitation experiment (Fig. 1a) was conducted by treating the roots with the same concentration of COS that was used in previous studies on CHIT elicitation (Tocci et al. 2011, 2013a; Brasili et al. 2014, 2016). By recording the xanthone content over time, two accumulation peaks were detected (Fig. 3), the first one at an early stage and the second one at a later stage, i.e., on days 5 and 25 post-elicitation. Brasili et al. (2014, 2016) investigated the combined effect of CHIT and overcrowding stress on both primary and secondary metabolism in *H. perforatum* root cultures and through an NMR-based metabolomics approach and ANOVA simultaneous component analysis (ASCA), they demonstrated that early responses are mainly caused by the elicitor, while the late responses are generated by the combined effect of the elicitor and the overcrowding stress due to the high root biomass growth in a confined environment. The impact of overcrowding stress clearly emerges by the observation of control roots, the xanthonic content of which progressively increases with culture duration (Fig. 3). The early response

leads to neosynthesis of almost all the analyzed xanthones, while in the xanthone profile corresponding to the late response, the dominant compound is paxanthone. In future applications of *H. perforatum* root cultures for the biotechnological production of xanthones, the choice of collecting the roots at a short- or long-time post-elicitation will have to be made on the basis of the molecules of interest.

In the second experiment (Fig. 1b), the effect of different COS concentrations on the xanthone content was investigated. The best results in terms of the total xanthone content were obtained with the highest COS concentration tested in this study (400 mg L^{-1}). This concentration is much higher than those used in most CHIT elicitation experiments, both on cell (Wiktorowska et al. 2010; Chakraborty et al. 2009; Ferri et al. 2009) and root cultures (Udomsuk et al. 2011; Sivanandhan et al. 2012; Shinde et al. 2009; Putalun et al. 2007). With 400 mg L^{-1} COS, the xanthone content reached very high levels (over 30 mg g^{-1} DW), which has never been obtained with CHIT in the previous studies (Tocci et al. 2011, 2012, 2013a; Brasili et al. 2014; Simonetti et al. 2016). It should be emphasized that FDA test showed that these COS concentrations, while causing visible symptoms of suffering to roots (Fig. 8), do not cause death, as opposed to CHIT, which even at lower concentrations cause PCD or necrosis in plant cells (Zuppini et al. 2004; Iriti et al. 2006) and in *H. perforatum* cultured roots (personal observation). Cytotoxicity of CHIT represents a limitation for biotechnological purposes, due to the dramatic morpho-anatomical alterations caused by this elicitor (Brasili et al. 2016) which make *in vitro*-cultured cells and organs non-reusable for subsequent production cycles. From the analysis of the individual xanthones, it appears that the administration of 400 mg L^{-1} COS stimulates the biosynthesis of all the analyzed xanthones, except mangiferin.

The effect of COS on *H. perforatum* cultured roots has been also investigated in terms of volatile organic compound (VOC) biosynthesis. In a previous study, we observed a remarkable increase in dimethylallyl-pyrophosphate (DMAPP) levels in *H. perforatum* CHIT-treated roots (Brasili et al. 2014). Since the ^{1}H-NMR analysis failed to reveal the presence of terpenoids, we performed both histochemical and headspace VOCs analysis of *H. perforatum* roots in this study, with the aim to investigate the presence of isoprenoids in root biomass after COS elicitation.

First, the histochemical analysis with Nadi reagent suggested the presence of isoprenoid compounds localized in secretory ducts of both control and treated roots. The presence of essential oils in secretory ducts was previously demonstrated only in the aerial parts of *H. perforatum*, including sepals, petals, stamens, leaf, and stem, but never in roots (Ciccarelli et al. 2001). Second, the GC-MS headspace analysis confirmed the presence of a wide

spectrum of isoprenoids in cultured roots. The lack of isoprenoids in the extracts previously analyzed by 1H-NMR (Brasili et al. 2014) was probably due to their volatilization during the Bligh-Dyer extraction, as the identified isoprenoids (mono- and sesqui-terpenes) have a low molecular weight corresponding to a high volatility.

It is well-documented that roots are able to synthesize and release VOCs in the rhizosphere, where act as key mediators in belowground biotic interactions (Delory et al. 2016). In this scenario, VOCs can have negative (phytotoxins, autoinhibition, and development of associations with parasitic plants) or positive effects (resistance to herbivores and root detection) on neighbouring plants, but also can affect plant growth directly (phytotoxin biosynthesis) or indirectly (alteration of soil chemistry, microbial populations, and nutrient availability) (Weston et al. 2012; Zeng 2014). The majority of the studies published so far focus on root VOC biosynthesis by three major plant models as Zea mays, Citrus spp., and Brassica spp. in response to nematodes and parasites (Delory et al. 2016). To our knowledge, it is the first study that deals with the biosynthesis of VOCs by *H. perforatum* roots in response to COS elicitation. The obtained results suggest that VOCs play a key role in mediating the interactions between *H. perforatum* root and soil organisms, especially fungi, since their biosynthesis resulted affected by COS, which are fungal elicitors.

As reported in our previous studies, xanthone-rich crude extracts obtained from *H. perforatum in vitro*-cultured roots elicited with CHIT exhibit a high antifungal activity (Tocci et al. 2011, 2012, 2013; Simonetti et al. 2016). In the present study, we observed that elicitation with 200 mg L^{-1} COS leads to a total xanthone content comparable to that obtained using CHIT at the same concentration; however, a higher antifungal activity was observed compared to the previous results. These results suggest that the extracts contain other metabolites with antifungal activity induced by COS, which may act additively or synergistically with xanthones. An antifungal activity of certain VOCs such as monoterpenes, monoterpenes hydrocarbons, sesquiterpenes, and diterpenes was previously demonstrated in other plant species (Badawy et al. 2017; Fraternale et al. 2016) and against other fungi such as Fusarium verticillioides, the major producer of mycotoxin in contaminated aliments (Dambolena et al. 2008).

Conclusions

For the first time in the present study, the elicitor effect of COS was tested on St. John's wort *in vitro* root cultures. The obtained results showed that COS are very effective elicitors, more

powerful of CHIT in stimulating the biosynthesis of xanthones in *H. perforatum* root cultures. They also showed a lower phytotoxicity that allows its usage at high concentrations. In addition to enhancing xanthone biosynthesis, COS caused significant changes in the production of VOCs. The obtained results suggest that xanthones and VOCs are involved in regulating the relationships between root and edaphic microorganisms, especially fungi.

Author contribution statement

AV conceived and designed experiments, analyzed data, and prepared figures and tables; AV, GDA, and CB performed experiments and the HPLC analysis of xanthones; EB, ECT, and EP carried out GC-MS headspace analysis; HY synthesized COS; AV, CB, GDA, EB, GS, and GP wrote the manuscript; GP coordinated and guided intellectually all process of the work; GP and GS made a critical revision of the manuscript. All the authors read and approved the manuscript.

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Compliance with ethical standards

Conflict of interest. The authors have declared that no competing interests exist.

Supplementary

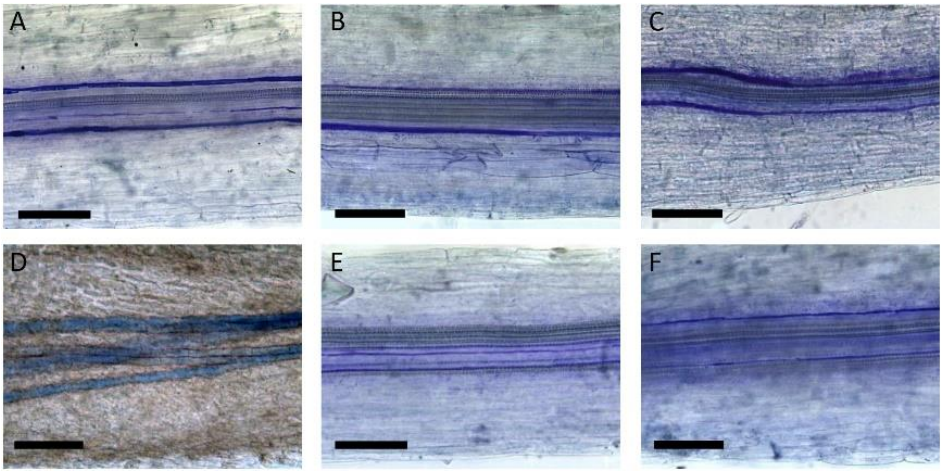

Fig. S1 *In vitro* roots of *H. perforatum* treated with Nadi reagent and observed under bright field. Control root (A) and roots elicited with 50 (B), 100 (C), 200 (D), 400 (E) and 800 (F) mg L^{-1} COS. Bars represent 200 µm.

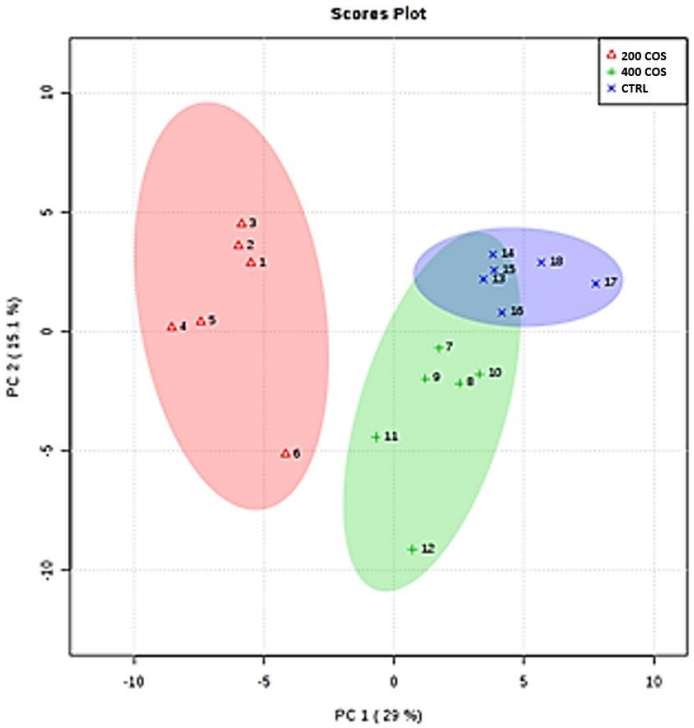

Fig. S2 PCA score plot of the *H. perforatum* roots analyzed by SPME-GC-MS methodology. Control samples (CTRL) are represented in blue; chitosan-treated samples are represented in red (200 mg L^{-1}) and in green (400 mg L^{-1}).

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Abbreviations chapter 3

CHIT	chitosan
COS	chitosan oligosaccharides
DW	dry weight
FW	fresh weight
HPLC	high performance liquid chromatography
IBA	indole butyric acid
MeJA	methyl jasmonate
ROS	reactive oxygen species
SA	salicylic acid

3. Response of *Hypericum perforatum* root cultures and *in vitro*-grown plantlets to chitosan oligosaccharides (COS), methyljasmonate (MeJA), salicylic acid (SA) and hydrogen peroxide (H_2O_2)

Badiali C · Brasili E · Iozia LM · Petruccelli V · Caparra MV · Di Giovenale A · Pasqua G · Valletta A

Department of Environmental Biology, Sapienza University of Rome, Piazzale Aldo Moro 5, 00185 Rome, Italy

Introduction

Being sessile and relatively immobile organisms, plants are unable to simply escape adverse environmental conditions. These organisms had to evolve around life's adversities instead, setting up reliable stress sensing mechanisms and adequate threat-specific responses, such as the synthesis of defensive secondary metabolites (Mazid et al. 2011).

Plants are sensible to both abiotic and biotic stress. The perception of a potential pathogen can involve signal molecules which can either be exogenous or endogenous (Namdeo et al. 2007; Petrov et al. 2012). A plant can perceive the attack of a fungus through sensing a broad range of molecules of fungal origin (lipids, proteins, nucleic acids, polysaccharides) called MAMPs (microbe-associated molecular patterns). Chitin derivatives coming from fungal cell walls activate plant responses. On the other hand, oligosaccharides generated by the fungal-mediated lysis of plant cell wall polysaccharides can also signal the pathogen's presence to the plant (Namdeo et al. 2007; Gadzovska Simic et al. 2014, 2015).

Molecules capable of inducing defense responses are known as "elicitors" (Namdeo et al. 2007). The discovery of the biological role of these molecules has had a high impact on both basic and applied research. Regarding biotechnological applications, elicitors can be exploited to induce or enhance the neosynthesis of secondary metabolites of human interest from cell and organ cultures (Namdeo et al. 2007; Gadzovska Simic et al. 2014).

After the stimulus is perceived, it needs to be transduced and amplified in order to influence gene expression and thus stimulate an array of responses, including the biosynthesis of defenses secondary metabolites. Different signalling molecules are involved in biotic stress responses such as oxygen peroxide (H_2O_2), salicylic acid (SA) and jasmonates (JAs) (Namdeo et al. 2007; Petrov et al. 2012; Yan et al. 2014; Goossens et al. 2016). SA and JAs are involved in distinct pathways (Bennett et al. 1994; Yan et al. 2014; Goossens et al. 2016). Systemic acquired resistance (SAR), which is due to biotrophic and virus infection, is

associated with SA accumulation and the upregulation of genes encoding for pathogenesis-related (PR) proteins. In contrast, induced systemic response (ISR), which is due to necrotrophic infection and pest attack, is dependent on JA and ethylene (ET) pathways and is not associated with PR gene expression. Otherwise, H_2O_2 plays a key role in the early stages of most stress-related routes, concurrently oxidizing the pathogen during the early response oxidative burst, acting as a signaling molecule activating genes involved in defense responses and reinforcing the plant cell wall (Apostol et al. 1989; Petrov et al. 2012).

Recent studies mainly focused on biosynthesis, transport and accumulation of defensive secondary metabolites in Solanaceae. They showed complex interactions between different organs of the plants (De Luca et al. 2000). Interestingly, certain metabolites such as nicotine have been proven to be induced at the leaf, produced at the root and then transported and accumulated in the leaves (Erb et al. 2009 and literature cited therein). The root / bud interactions implicated in the biosynthesis of secondary metabolites in response to environmental stimuli is a field of study of great interest and still largely unexplored. Among medicinal species St. John's wort (*Hypericum perforatum* L.) is one of the most studied, mainly because it produces bioactive metabolites of pharmaceutical interest that accumulate in its shoots (naphthodianthrones and phloroglucinols) (Gadzovska Simic et al. 2014). Recently, interest has also aimed at xanthones, secondary metabolites that proved to possess several interesting medicinal properties (Pinto et al. 2005). *H. perforatum* xanthones, which specifically accumulate in the root, showed a remarkable antifungal activity against certain human fungal pathogens, such as *Cryptococcus neoformans* (Tocci et al. 2011), *Malassezia furfur* (Simonetti et al. 2015), *Candida albicans*, *Trichophyton mentagrophytes*, and *Microsporum gypseum* (Badiali et al. 2018). The antimicrobial activity of xanthones from *H. perforatum* suggest their involvement in plant's defense mechanisms (Tocci et al. 2013a-b; Badiali et al. 2018).

Recent studies have also shown that xanthone production in root cultures of St. John's wort can be induced or amplified by fungal elicitors such as chitosan (Brasili et al. 2016; Badiali et al. 2018). Conversely, the effects of other molecules known to act as elicitors in other species (SA, JA and H_2O_2) have not been thoroughly investigated yet.

In this context, *H. perforatum* root cultures and seedlings obtained in vitro were employed as a model system to study the interaction between roots and shoots in the biosynthesis of bioactive secondary metabolites. Xanthone and hypericin content was investigated in roots and shoots respectively.

Materials and methods

Root cultures

Adventitious roots of *H. perforatum* were obtained as previously described in Valletta et al. (2016). Liquid root cultures were established inoculating 0.250 g fresh weight (FW) of roots in 100 mL flasks containing 50 mL half-strength MS basal salts and vitamins medium (Murashige and Skoog 1962) supplemented with 1 mg L^{-1} Indole-3-butyric acid (IBA) and 15 g L^{-1} sucrose. Flasks were shaken at 100 rpm at 26 ± 1 °C and maintained in continuous dark until harvest. MS medium and sucrose were purchased from Duchefa Biochemie (Haarlem, The Netherlands) and IBA from Sigma-Aldrich (Milan, Italy).

Source, preparation and *in vitro* germination of seeds

H. perforatum capsules were harvested in June 2016 and 2017 from the same population located in Marcigliana Natural Park (Rome). They were dried at room temperature and the seeds were collected and stored at room temperature until use. Before the sowing under *in vitro* conditions, seeds were enclosed in bags of filtration tissue and soaked in distilled water for 3 hours at room temperature. Then, they were sterilized in 70% ethanol for 3 min, rinsed in distilled water and sterilized again in commercial sodium 15% hypochlorite (active chlorine 4.9%) containing 0.1% Tween (Sigma-Aldrich, Milan, Italy) for 20 min. At the end three rinses with sterile water were performed. After the sterilization, seeds were inoculated in 100 mL flasks containing 50 mL half-strength MS basal salts and vitamins medium supplemented with 15 g L^{-1} sucrose. Flasks were shaken at 100 rpm at 26 ± 1 °C and maintained in 16/8 hours light/dark condition; plantlets where subcultured every 20 days until a sufficient amount of biomass was obtained. Liquid plantlet cultures for the experiment were established inoculating 2 g FW of biomass (roots and shoots) and cultured in the same medium and conditions described above.

Priming and elicitation protocols

Root and plantlet cultures were primed with methyl jasmonate (MeJA) (Sigma-Aldrich, Milan, Italy), salicylic acid (SA) or H_2O_2 on day 7 of culture using a final concentration of 100 μM, 25 μM and 100 mM, respectively. A 4.5 mg L^{-1} stock solution of MeJA and a 1.7 g L^{-1} stock solution of SA were prepared to add 250 μL to each flask. A 30% commercial solution of H_2O_2 was used to obtain a final concentration of 100 mM. Roots and plantlets were elicited on day 8 of culture with 400 and 200 mg L^{-1} chitosan oligosaccharides (COS) obtained as described in Badiali et al. (2018) and dissolved in deionized water. In the

experiments on the combined effect of H_2O_2 and COS, the latter were used at a concentration of 200 mg L^{-1}. Regarding H_2O_2 experiment, the final COS concentration used for both root cultures and plant roots was 200 mg L^{-1}. An 80 g L^{-1} stock solution was prepared to add 250 µL to each flask (Fig. 1). The stock solutions were sterilized with a 0.2 µm syringe filter before addition to the liquid culture medium. Control samples were added with 250 µL of deionized sterile water. Control and roots treated with COS, MeJA and SA were collected 25 days after the elicitation with COS (day 33 of culture). Control and roots treated with COS and H_2O_2 were collected 7 days after COS elicitation (day 15 of culture) as well as control and plantlets treated with COS, MeJA, SA and H_2O_2.

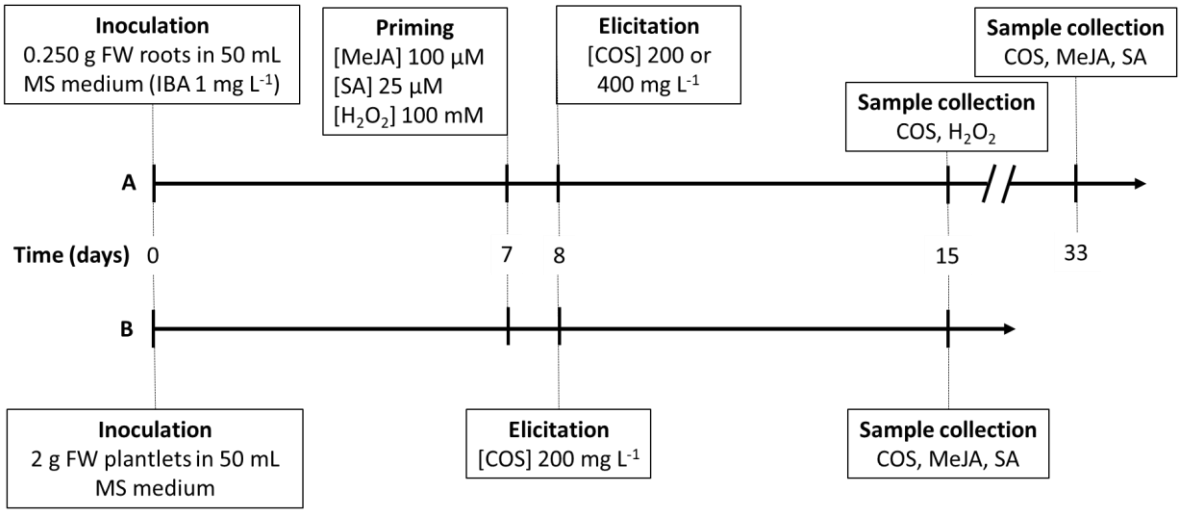

Fig. 1 Diagram representing the elicitation protocols. A) Root elicitation protocol and B) plantlets elicitation protocol.

Xanthone quantification

The roots were dried in an oven at 70 °C for 48 hours and then powdered with pestle and mortar. Three consecutive extractions were performed (each 24 h) with methanol at a ratio biomass/solvent of 100:5 (mg/mL). The extracts were dried with a rotary evaporator (Buchi, Milan, Italy) at 35 °C and re-dissolved in HPLC-grade methanol (Carlo Erba, Milan, Italy) at the ratio initial biomass/solvent of 100:1 (mg/mL). The extracts were analyzed by high-performance liquid chromatography (HPLC), as described by Tocci et al. (2013a) and Valletta et al. (2016). Following xanthones were identified and quantified: mangiferin; 1,3,6,7-tetrahydroxyxanthone; 1,3,5,6-tetrahydroxyxanthone; cadensin G; 1,7-

dihydroxyxanthone; toxyloxanthone; paxanthone and 5-O-methyl-2-deprenylrheediaxanthone.

Statistical analysis

A total of 3 biological replicates of samples for each treatment were analyzed by HPLC. All measurements were made at least in triplicate and the results were expressed as means ± SD. Statistical analysis was carried out using SigmaPlot 13.0. One-way analysis of variance (ANOVA), followed by Holm-Sidak test, was applied to test differences between groups. Statistical significance of the results was also evaluated by paired Student's *t* test, and differences with a p value ≤ 0.05 were considered significant.

Results

Elicitation of root cultures with COS, MeJA, SA or H_2O_2 used alone or in combination

COS, MeJA, SA and H_2O_2 were used to treat root cultures of *H. perforatum*. HPLC analysis performed on roots collected on day 33 of culture showed that the elicitation with COS significantly enhances xanthone production in terms of total xanthones compared with the control (27.5 and 6.2 mg g^{-1} DW, respectively). COS treatment also induced the highest increase compared with MeJA and SA both used alone or in combination with COS (Fig. 2). Among treatments, COS used alone caused the highest increase in xanthone production (+343% in comparison with control). MeJA and SA used alone caused significant but very low increases in xanthone biosynthesis (+27 and +18% in comparison with control, respectively) and when used in combination with COS they seem to largely reduce the COS effect (+87 and + 37% in comparison with control, and -58 and -46% in comparison with COS-treated, respectively). In the roots elicited with COS, all analyzed xanthones were produced at much higher levels than roots treated with MeJA or SA, except for Mang and Dihydroxy. The highest amounts of Mang were detected in control roots and in samples treated with MeJA and SA used alone. The highest production of Rheedia was detected in samples treated with SA used in combination with COS. Toxy was detected in each sample except for SA+COS treated ones. Dihydroxy was detected only in samples treated with MeJA used in combination with COS only (Fig. 3).

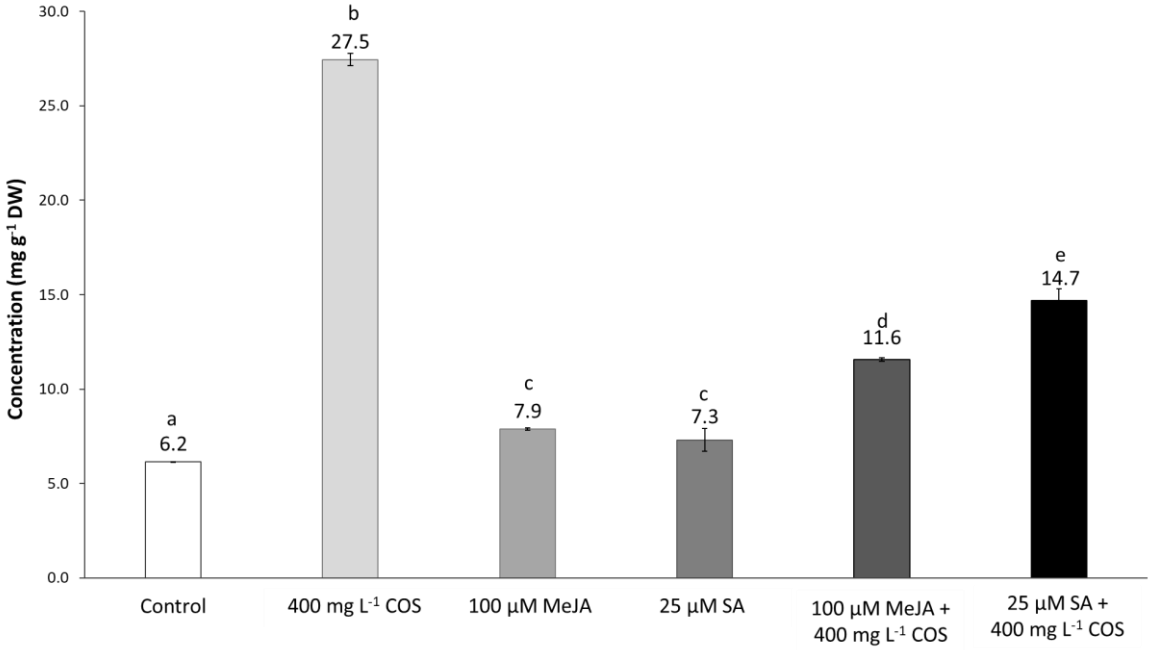

Fig. 2 Total xanthone concentration in root cultures of *H. perforatum* not subjected to priming and elicitation (control) or treated with COS, MeJA or SA used alone or in combination. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Different letters represent significant differences between samples ($p \leq 0.05$).

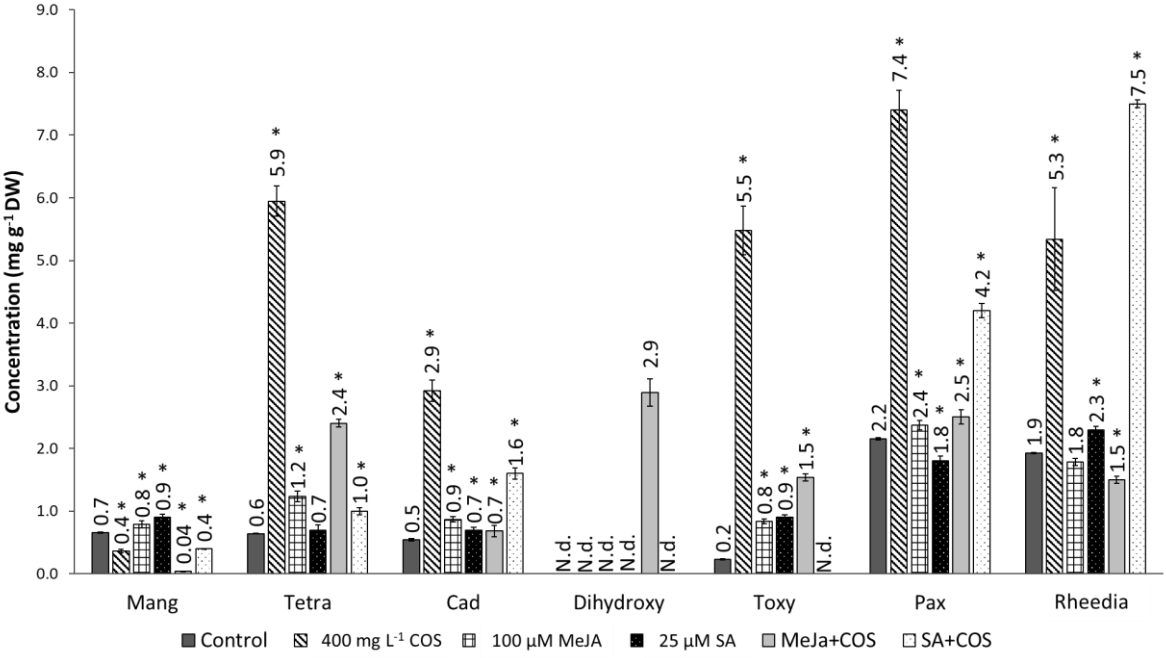

Fig. 3 Xanthone content in *H. perforatum* root cultures not subjected to priming and elicitation (control) or treated with COS, MeJA or SA used alone or in combination. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars

represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Asterisks indicate that the differences between roots and control roots are statistically significant ($p < 0.05$). Mang: mangiferin; Tetra: 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone; Cad: cadensin G; Dihydroxy: 1,7-dihydroxyxanthone; Toxy: toxyloxanthone; Pax: paxanthone; Rheedia: 5-O-methyl-2-deprenylrheediaxanthone; N.d.: non-detected.

In H_2O_2-COS experiment, H_2O_2 used alone showed a significantly lower effect in eliciting xanthone production compared to COS (2.5 and 3.2 mg g^{-1} DW, respectively). However, when used in combination the effect on xanthone production was higher than those of COS and H_2O_2 used alone (5.0 mg g^{-1} DW) (Fig. 4). In the roots elicited with COS, all analyzed xanthones were produced at much higher levels than roots treated with H_2O_2 and control roots. H_2O_2 used alone induced a higher production of Mang and Pax compared with control samples. The highest amounts of Mang were detected in samples treated with H_2O_2 used alone and in roots treated with H_2O_2 and COS used in combination (1.44 and 1.37 mg g^{-1} DW, respectively). The highest production of all xanthones was detected in samples treated with H_2O_2 used in combination with COS except for Rheedia which was higher in COS treated roots (0.47 mg g^{-1} DW).

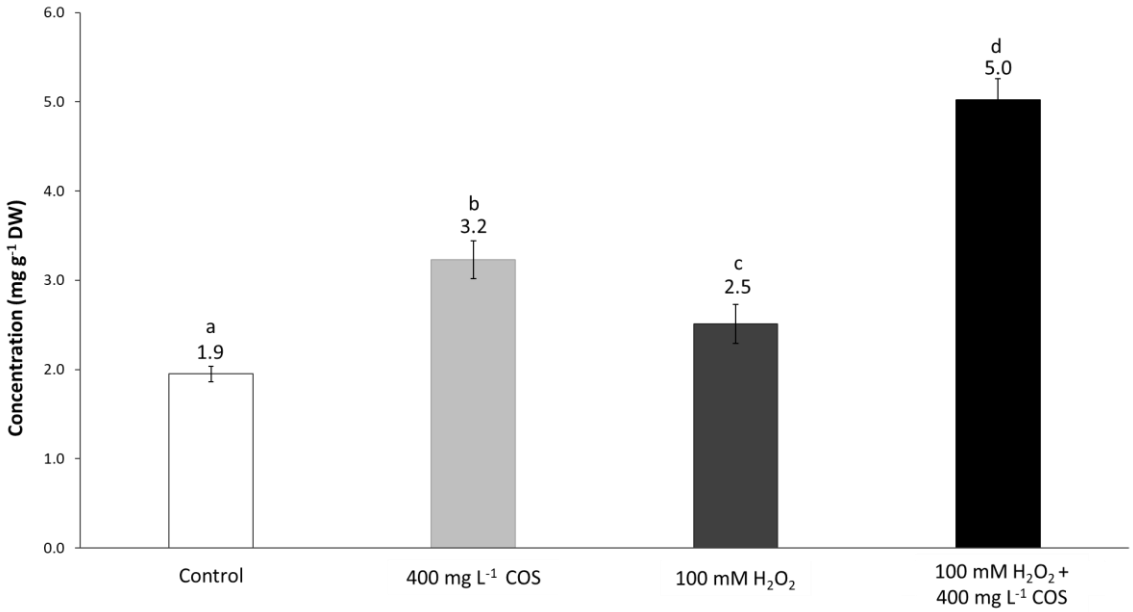

Fig 4. Total xanthone concentration in root cultures of *H. perforatum* not subjected to priming and elicitation (control) or treated with COS or H_2O_2 used alone or in combination. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three

independent biological replicates. Different letters represent significant differences between samples ($p \leq 0.05$).

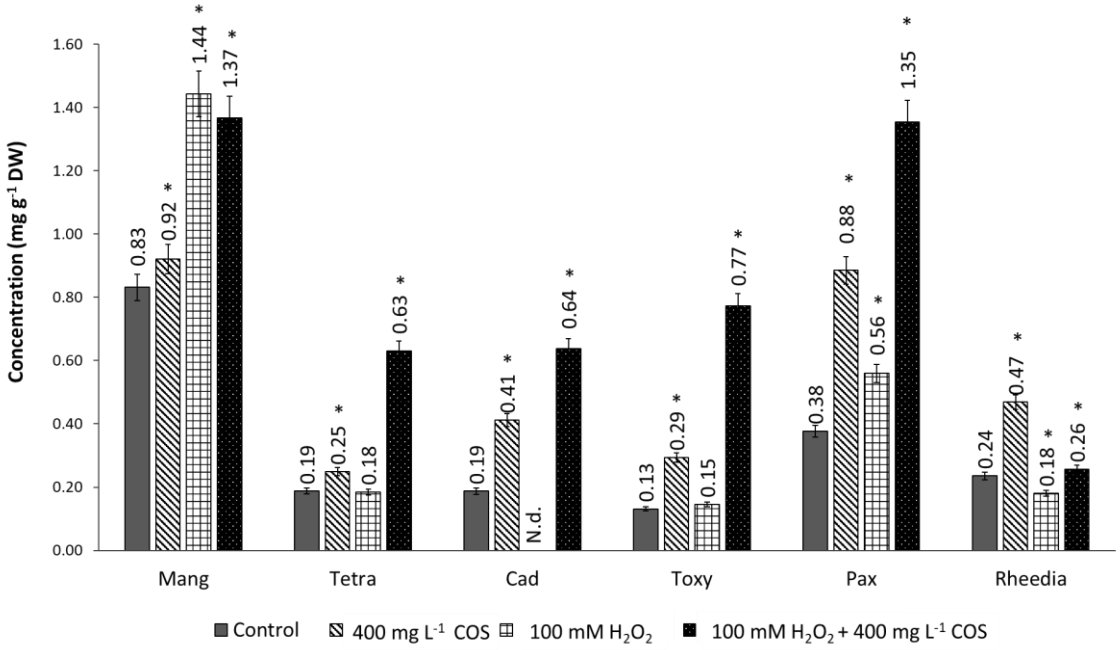

Fig. 5 Xanthone content in *H. perforatum* root cultures not subjected to priming and elicitation (control) or treated with COS or H_2O_2 used alone or in combination. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Asterisks indicate that the differences between elicited roots and the corresponding control roots are statistically significant ($p < 0.05$). Mang mangiferin; Tetra 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone; Cad cadensin G; Dihydroxy 1,7-dihydroxyxanthone; Toxy toxyloxanthone; Pax paxanthone; Rheedia 5-O-methyl-2-deprenylrheediaxanthone; N.d. non-detected.

Elicitation of *in vitro*-grown plantlets with COS, MeJA, SA or H_2O_2 used alone or in combination

COS, MeJA, SA and H_2O_2 were used to treat *H. perforatum in vitro*-grown plantlets. Chemical analysis was performed on roots collected on day 15 of culture. HPLC analysis showed that MeJA elicitation significantly enhanced xanthone production in terms of total xanthones compared with the control (3.3 and 1.7 mg g^{-1} DW, respectively) and induced the highest increase compared with COS and SA used alone or in combination with COS (Fig. 6). In the roots elicited with MeJA used alone, all analyzed xanthones were produced at

higher levels than roots treated with COS or SA, except for Toxy and Pax. The highest amount of Toxy was detected in samples treated with MeJA used in combination with COS. The highest production of Pax was detected in samples treated with MeJA used in combination with COS. Tetra was detected in each sample except for the ones treated with SA. Dihydroxy was detected in samples treated with MeJA used in combination with COS only (Fig. 7).

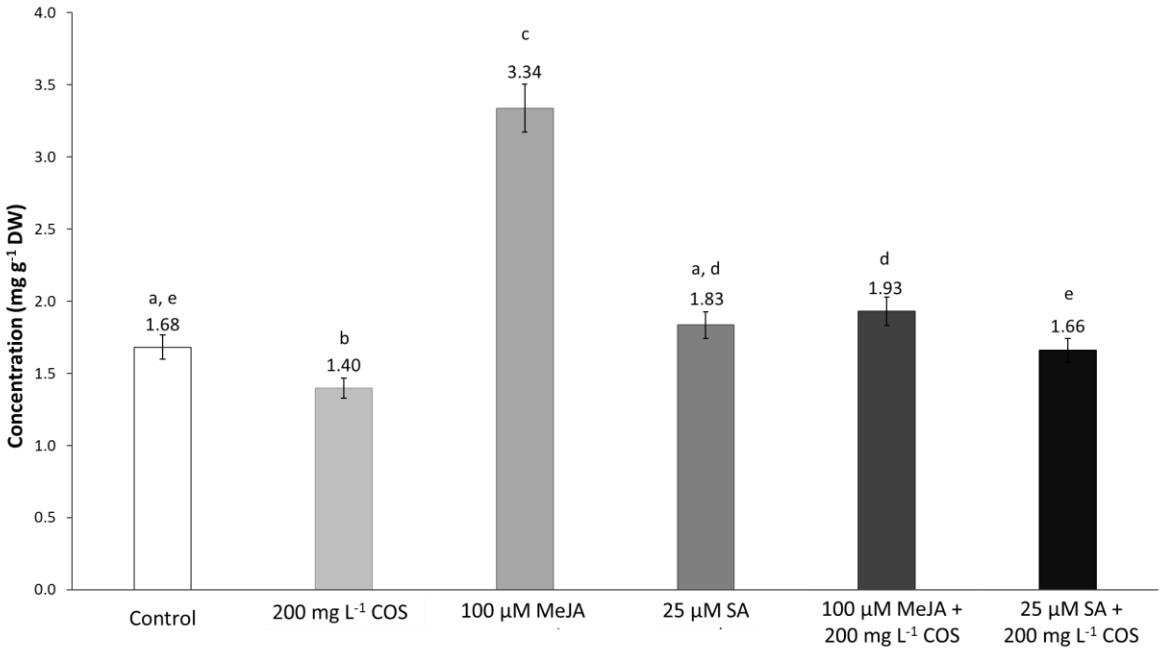

Fig. 6 Total xanthone concentration in roots of *in vitro*-grown plantlets of *H. perforatum* not subjected to priming or elicitation (control) or treated with COS, MeJA or SA. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Different letters represent significant differences between samples ($p \leq 0.05$).

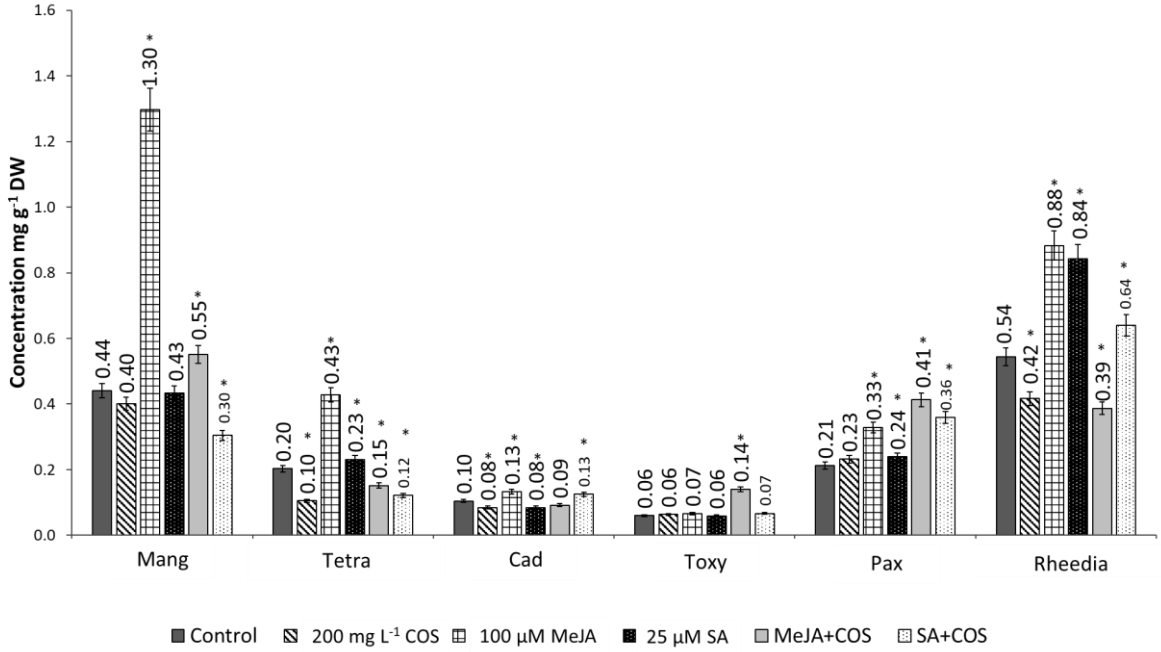

Fig. 7 Xanthone content in roots of *in vitro*-grown plantlets of *H. perforatum* not subjected to priming or elicitation (control) or treated with COS, MeJA or SA used alone or in combination. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Asterisks indicate that the differences between elicited roots and the corresponding control roots are statistically significant ($p < 0.05$). Mang mangiferin; Tetra 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone; Cad cadensin G; Dihydroxy 1,7-dihydroxyxanthone; Toxy toxyloxanthone; Pax paxanthone; Rheedia 5-O-methyl-2-deprenylrheediaxanthone.

The diagram in Fig. 8 shows the hypothetical interactions between rott and shoot in the perception of the elicitor.

Fig. 8 Putative interaction between shoot and root in elicitor perception. The presence of the shoot seems to be necessary for MeJA perception, causing then xanthone production in the roots in a cross-talk between the shoot and the root.

The treatment with H_2O_2 enhanced COS eliciting effect when they were used in combination, causing a significant increase in xanthone content compared to control (2.22 and 1.77 mg g^{-1} DW, respectively) (Fig. 9). When used alone, H_2O_2 caused a significant decrease compared to control (1.31 and 1.77 mg g-1 DW, respectively). In the roots elicited

with H_2O_2 used in combination with COS, Mang, Cad, Pax and Biyou were produced at higher levels than in roots treated with COS or H_2O_2 alone. Toxy production was the lowest in COS+H_2O_2 samples. Kielc was detected in each sample, although at low levels, except for those treated with COS+H_2O_2 (Fig. 10).

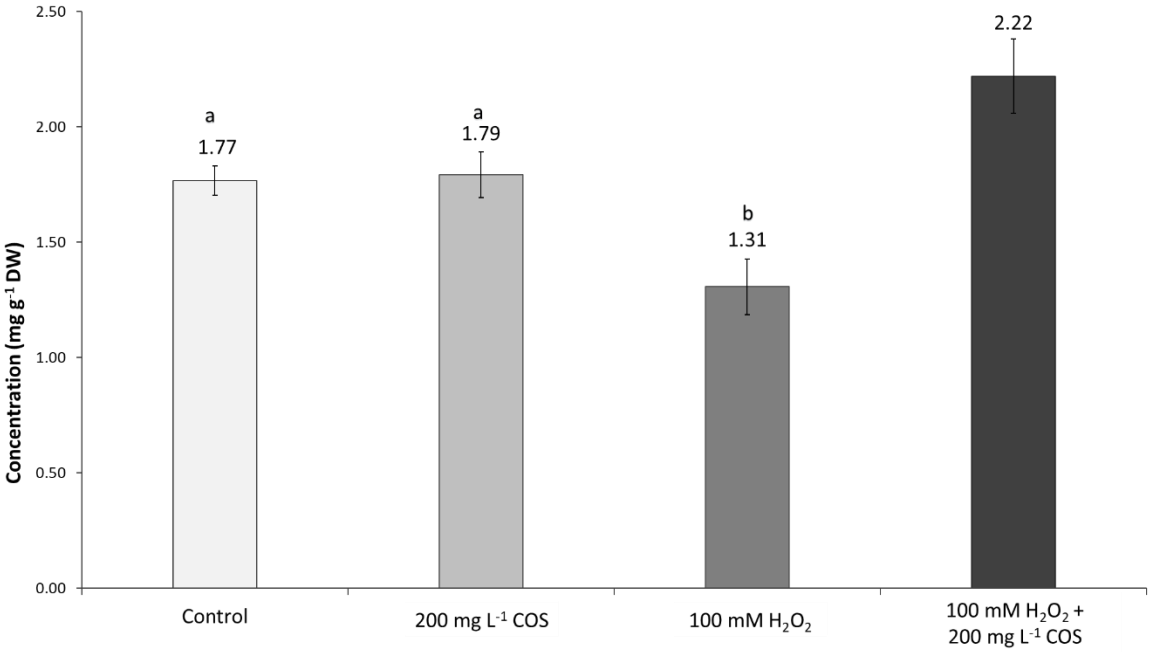

Fig. 9 Total xanthone concentration in roots of *in vitro*-grown plantlets of *H. perforatum* not subjected to priming and elicitation (control) or treated with COS or H_2O_2 used alone or in combination. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Different letters represent significant differences between samples ($p \leq 0.05$).

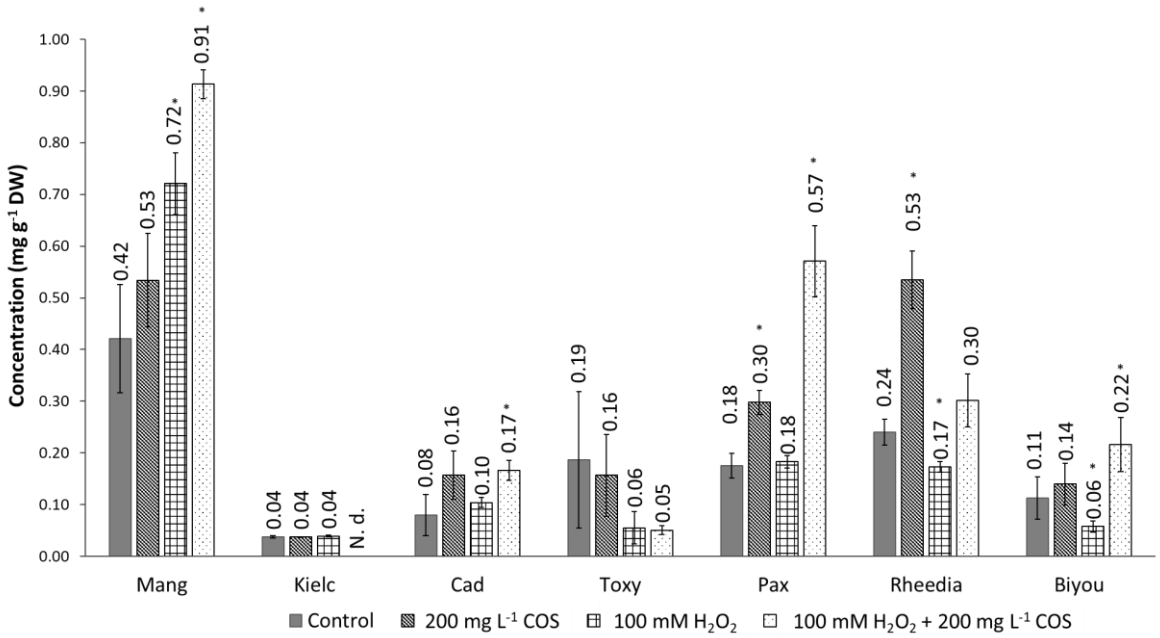

Fig. 10 Xanthone content in roots of *in vitro*-grown plantlets of *H. perforatum* not subjected to priming and elicitation (control) or treated with COS or H_2O_2 used alone or in combination. The eliciting concentrations used are the same for both single and combined administration. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Asterisks indicate that the differences between elicited roots and the corresponding control roots are statistically significant ($p < 0.05$). Mang mangiferin; Cad cadensin G; Dihydroxy 1,7-dihydroxyxanthone; Toxy toxyloxanthone; Pax paxanthone; Rheedia 5-O-methyl-2-deprenylrheediaxanthone.

Discussion

H. perforatum is a medicinal plant spread and cultivated all over the world. Its use in medicine has an antique tradition and nowadays it is mainly used as remedy against depression, anxiety and neurovegetative disorders (Gadzovska Simic et al. 2014). Clinical studies have demonstrated that the bioactive secondary metabolites mainly responsible for the antidepressant activity of *H. perforatum* are naphthodianthrones (hypericins) and phloroglucinols (hyperforins), which are specifically biosynthesised and accumulated in the aerial organs of the plant (Gadzovska Simic et al. 2014; Russo et al. 2014). It has been proposed that the antidepressant activity of these metabolites is due to their ability to inhibit the reuptake of some neurotransmitters, including serotonin, dopamine, glutamate, noradrenalin and GABA (Tian et al. 2014). Although the research has mainly focused on these compounds (Kasper et al. 2010), in recent years attention has also been paid to other bioactive metabolites such as volatile compounds including essential oils, flavonoids,

tannins, xanthones and other phenylpropanoids. We recently demonstrated a remarkable antifungal activity of xanthones against several human pathogenic fungi (Tocci et al. 2011, 2013a-b; Simonetti et al. 2016). This finding is particularly interesting in the context of the increasing demand for new antifungals due to the alarming emergence of drug resistance caused by the intensive use of the conventional antifungals as azoles (Price et al. 2015).

This work had both application and basic purposes. The first objective was to investigate the effect of various biotic elicitors (COS, MeJA, SA and H_2O_2) in order to increase the production of xanthone in *H. perforatum*. The second objective was to elucidate the influence of shoot/root interaction on elicitor perception already reported in other species but not observed in *Hypericum* spp. yet (Erb et al 2009 and literature cited therein). The tested elicitors and their concentration have been chosen due to their involvement in plant stress responses and to their established effect as elicitors on a number of plant species (León et al 1995; Shin and Schachtman 2004; Gadzovska et al. 2013; Wang et al. 2015; Badiali et al. 2018).

Results showed that COS were the most effective in stimulating xanthone production in root cultures of *H. perforatum* compared to other biotic elicitors tested in this work. The best results were obtained with 400 mg L-1 COS, which caused a 343% increase over the control; lower but significant increase were induced by MeJA, SA and H_2O_2 used alone. When used in combination on root cultures these elicitors behaved differently. Both MeJA and SA reduced the COS effect, probably due to the activation of other metabolic pathways or due to a higher response. Correspondingly, also a more extended oxidative burst could be associated with a higher consumption of xanthones. Franklin and co-workers (2009) showed that in *H. perforatum* xanthones could act as antioxidants against reactive oxygen species generated during the first steps of plant defense responses (Fig. 2). The treatment with H_2O_2 enhanced the effect COS, inducing an increase in the xanthone concentration. This shows an additive effect, which was determined by the highest xanthone levels, and determining the highest xanthone biosynthesis obtained in this experiment. H_2O_2 exogenously administered in combination with COS, may induce in the cell a higher xanthone production (compared to COS alone) to counteract the negative effects of both endogenous (induced by COS) and exogenous (administered H_2O_2) ROS. It should be stressed that H_2O_2 was administred at a concentration (100 mM) remarkably higher compared to the physiological levels (from 0.07 to 130 μmol g^{-1} FW) (Cheesman 2006 and literature cited therein). Non-physiological high concentrations of H_2O_2 have been previously used to induce defense responses (León et al 1995). When used alone it induced a low but significant increase in xanthone production (Fig. 4).

In the first experiment, the analysis of the individual xanthones showed that COS administration stimulated the highest biosynthesis of all analyzed xanthones except for Mang, Dihydroxy (obtained in MeJA+COS-elicited cultures only) and Rheedia (Fig. 3). In the second experiment, H_2O_2+COS-treated samples showed the largest increase of all analyzed xanthones except for Mang and Rheedia (Fig. 5).

In regard to the *in vitro* experiments with plantlets, MeJA showed to be effective in eliciting the xanthone production in plant roots. Contrarily to its effect on root cultures, xanthone content was +99% compared to control. The presence of the shoot seems to be necessary for MeJA perception, causing then xanthone production in the roots in a cross-talk between the shoot and the root. Little information is available about the organ responsible for MeJA perception although, being a volatile molecule, involved in organ-to-organ and plant-to-plant signaling, it has been hypothesized that aerial organs are the main sites of perception (Erb et al. 2009 and literature cited therein). It was also argued that after perception in aerial organs, the signal is transferred to other plant districts including the root, where the defense response is activated. Another model considers a positive feedback loop of jasmonates (Sasaki et al. 2001; Wasternack and Hause 2013): the hypothesis is that both shoots and roots can perceive jasmonates but shoots only are able to amplify the signal needed for a defense response; this could explain why root cultures did not respond to MeJA elicitation.

MeJA was the most effective elicitor to induce xanthone biosynthesis in the root of *in vitro* plants, while COS have proved the least effective causing even a compared to control (-88%). When used in combination, COS and MeJA appeared to be antagonists also in plant roots. Interestingly, comparing MeJA+COS effect in root cultures and in *in vitro*-grown, a specular behaviour has been observed: in root cultures the most effective elicitors were COS, and their effect was lowered by MeJA, while in plants the best elicitor was MeJA, whose effectiveness was reduced by COS. A simplified diagram of the putative shoot-root interaction in elicitor perception and xanthone biosynthesis induction is proposed in Fig. 8.

SA used alone induced a non-significant increase in xanthone production. Xanthone production obtained with SA+COS was significantly lower than that obtained wits SA alone (Fig. 6), differently to what observed in root cultures, but showing again an antagonistic effect. From individual xanthone analysis, MeJA resulted as the most effective elicitor in stimulating the biosynthesis of all xanthones except for Toxy and Pax (Fig. 7). Results of H_2O_2 treatment on plant roots showed that when used alone it caused a significant decrease in the xanthone biosynthesis. When used in combination with COS they had an additive effect (Fig. 9) as showed in experiments on root cultures (Fig.4). From individual xanthone analysis H_2O_2+COS samples resulted as the most effective treatment in stimulating Mang,

Cad, Pax and Biyou biosynthesis (Fig. 10). The obtained results seem to show the strongest response to COS in root cultures and to the other tested elicitors in plant roots. H_2O_2 showed a similar behaviour either in root cultures and plant roots.

Conclusions

In the present study, the effect of four biotic elicitors was evaluated on St. John's wort root cultures and *in vitro*-grown plants. The results showed that COS are the most effective elicitors in stimulating the biosynthesis of xanthones in *H. perforatum* root cultures while MeJA is the most effective in stimulating the biosynthesis of xanthones in roots of *in vitro*-grown plants. This work gives interesting hints for further investigations on plant physiology, and its results are in addition to only few others available on the interaction between plant organs. The road ahead is still long to clarify the signaling mechanisms underlying these interactions. to clarify the signaling mechanisms underlying these interactions.

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Abbreviations chapter 4

COS	chitosan oligosaccharides
DW	dry weight
FW	fresh weight
HPLC	high performance liquid chromatography
IAA	indole acetic acid
IBA	indole butyric acid
ROS	reactive oxygen species

4. Effect of cadmium and arsenic on xanthone production in *Hypericum perforatum* root cultures

Introduction

The rapid growth of the world population and the intensification of anthropogenic activities has led to an increase in environmental contamination with a wide range of chemicals. Particularly worrying is the contamination by toxic metals, which can be absorbed by plants and then spread through trophic networks, with negative repercussions on both animal and human health.

In China 2.9% of the agricultural land (corresponding to about 4 million hectares) was found to be highly contaminated by heavy metals (Su 2014). Soil contamination is also widespread in Europe, covering 58% of the cultivated land, with concentration values of toxic metals above the thresholds recommended and applied by UNEP (Tòth et al. 2016). Although plants activate several defense mechanisms which control uptake, accumulation and translocation of heavy metals in order to detoxify them (Srivastava et al. 2004), heavy metals are often found in shoots, leaves, flowers, or, worse, seeds and fruits (Muchuweti et al. 2006; Unterbrunner et al. 2007; Shaheen et al. 2016). One common strategy is preventing the entrance of heavy metals into root cells by trapping them in the apoplast by detoxifying them via chelate complex formation (Watanabe and Osaki 2002) or to anionic groups of cell walls (Dalla Vecchia et al. 2005; Rascio et al. 2008). Most of the heavy metal amount that enters the plant is then kept in root cells, where it is detoxified by complexation with amino acids, organic acids or metal-binding peptides (e.g. phytochelatins) and/or sequestered into vacuoles (Salt and Rauser 1995; Piechalak et al. 2002). These trapping strategies protect the leaf tissues from damage.

High toxic metal concentrations cause various and different effects on plants both physiological and morpho-anatomical. They interfere with enzyme activity, metabolism of essential elements (Dong et al. 2006), and membrane integrity (Gadallah 1999); reduce photosynthesis, water and nutrient uptake; cause chlorosis, growth inhibition, browning of root tips, and death (Yadav 2010 and literature cited therein). Roots, being in direct contact with soils, are the most and first affected organ which show alterations both in their normal hormonal metabolism and in the development and morpho-anatomical differentiation, with damage that affects the growth of the entire plant.

In *Arabidopsis thaliana* and *Oryza sativa* it has been shown that both cadmium (Cd) and arsenic (As), toxic elements frequently present in polluted soils, express their toxicity by

altering both biosynthesis and transport of auxins, fundamental phytohormones for plant organogenesis (Ronzan et al. 2018; Fattorini et al. 2017). The correct distribution, carried out both through transport and conversion of the specific indole-3-butyric acid precursor (IBA) into its chemically active form indol-3-acetic acid (IAA), is required in various processes such as the genesis, development and maintenance over time of a functional root system (Strader et al. 2010). Moreover, effects of Cd on secondary metabolism were demonstrated in several species such as *Catharanthus roseus* (Zheng and Wu 2004), *Phyllanthus amarus* (Rai et al. 2005), *Brassica juncea* (Ahmad et al. 2016). To date, little is known about the impact of As on plant secondary metabolism.

Many species that survive in soils characterized by high heavy metal concentrations behave as "excluders", they retain and detoxify most of the heavy metals in the root tissues, with a minimized translocation to the leaves (Hall 2002). Otherwise, the term "hyperaccumulator" is used for plants which actively accumulate large amounts of one or more heavy metals from the soil and which translocate and accumulate them in aerial organs at concentrations hundreds-fold higher than non-hyperaccumulating species. These plants show no symptoms of phytotoxicity (Reeves 2006 and literature cited therein), and due to this, they could be more dangerous for human health especially in case of crops and medicinal plants. Among hyperaccumulating plants, there are species of numerous families such as Brassicaceae, Poaceae, Asteraceae, Fabaceae (Reeves et al 2006 and literature cited therein) and Hypericaceae including *H. perforatum* (Pavlova et al. 2015).

Materials and methods

Root cultures

Adventitious roots of *H. perforatum* were obtained as described in Valletta et al. (2016). Liquid root cultures were established inoculating 0.250 g fresh weight (FW) of roots in 100 mL flasks containing 50 mL half-strength MS basal salts and vitamins medium (Murashige and Skoog 1962) (Duchefa Biochemie, Haarlem, The Netherlands) supplemented with 15 g L^{-1} sucrose (Duchefa Biochemie). Flasks were shaken at 100 rpm at 26 ± 1 °C and maintained in continuous dark until harvest.

Treatment with Cd and As

Root cultures were treated with cadmium (Cd) and arsenic (As) on day 8 of culture using a final concentration of 50, 100 and 200 μM (Fig. 1). The stock solutions were sterilized with a 0.2 μm syringe filter before being added to the liquid culture medium. Control samples

were added with 500 µL of deionized sterile water (the same volume as treatment solution). Control and roots treated with toxic metals were collected 7 days after the treatment (day 15 of culture).

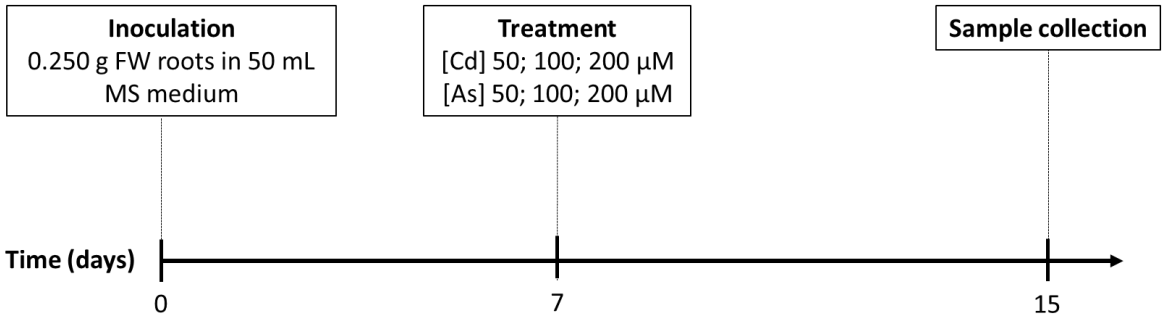

Fig. 1 Diagram representing the protocol of heavy metal treatment experiment.

Xanthone quantification

The root samples were dried at 70 °C for 48 hours and then powdered. Three consecutive extractions were carried out, one every 24 h, with methanol 100% (100 mg of dried root biomass in 5 mL). The raw extracts were dried with a rotary evaporator (Buchi, Milan, Italy) at 35 °C and re-dissolved in HPLC-grade methanol (Carlo Erba, Milan, Italy) (100 mg initial biomass in 1 mL). The chemical analyses were performed by high-performance liquid chromatography (HPLC), as described by Tocci et al. (2013) and Valletta et al. (2016). Eight xanthones were identified and quantified: mangiferin; 1,3,6,7-tetrahydroxyxanthone; 1,3,5,6-tetrahydroxyxanthone; kielcorin; cadensin G; toxyloxanthone; paxanthone; 5-O-methyl-2-deprenylrheediaxanthone and biyouxanthone.

Statistical analysis

Three biological replicates for each treatment were analyzed by HPLC. All measurements were made at least in triplicate and the results were expressed as means ± SD. Statistical analysis was carried out using SigmaPlot 13.0. One-way analysis of variance (ANOVA), followed by Holm-Sidak test, was applied to test differences between groups. Statistical significance of the results was also evaluated by paired Student's *t* test, and differences with a p value ≤ 0.05 were considered significant.

Results

Administration of different Cd and As concentrations to root cultures

Cd and As were used to treat root cultures of *H. perforatum*. HPLC analysis performed on roots collected on day 15 of culture showed that all Cd treatments (50, 100 and 200 μM) induced a decrease in the xanthone content compared to control (1.67, 1.40, 1.25 versus 1.75 mg g^{-1} DW, respectively) (Fig. 2). Samples treated with 50 μM Cd differences in xanthone content were no significant, while significant differences were caused by 100 and 200 μM Cd. Only the highest concentration of As (200 μM) caused a significant increase in xanthone production (+46% in comparison with control), while lower concentrations (50 and 100 mM) did not cause significant effects (Fig. 2). Significant decreases in xanthone content were caused by 100 and 200 mM Cd (-20 and -29%, respectively). In the roots treated with Cd, all analyzed xanthones were produced at equal or lower levels than non-treated roots except for Biyou in samples treated with Cd 50 μM. The differences between non-treated and treated samples as regards Mang production were non-significant in each sample. Tetra was detected in each sample except for As 100 μM. Kielc was detected in control, As 100 μM and As 200 μM samples. The highest production of Rheedia was detected in control samples only. (Fig. 3).

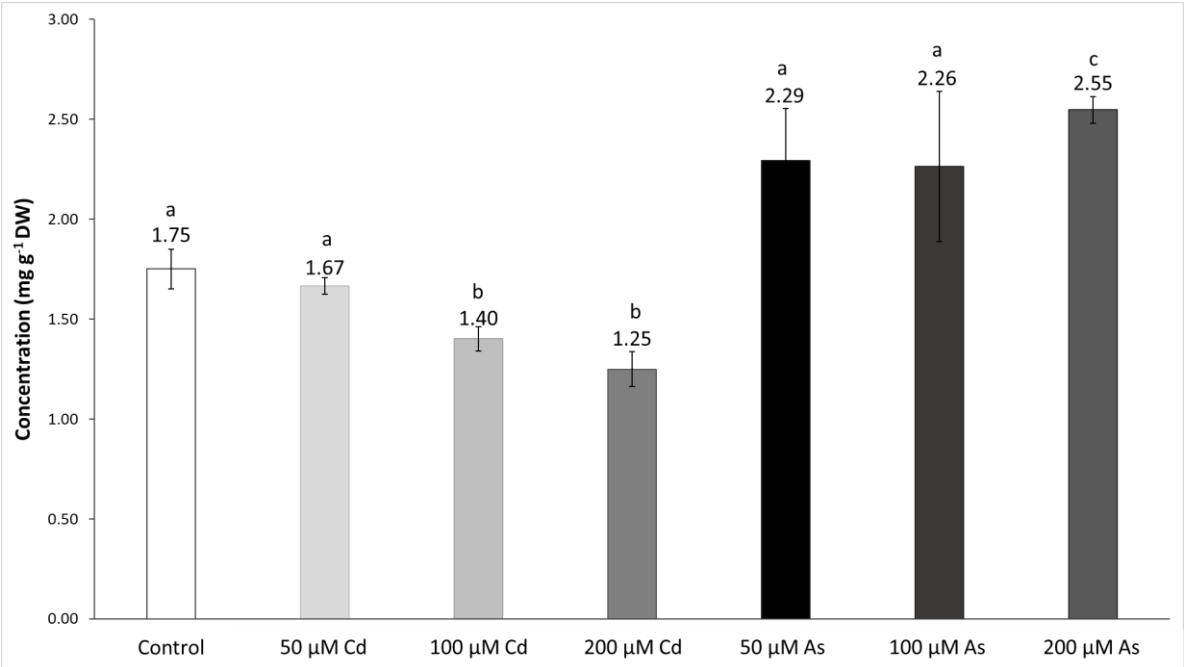

Fig. 2 Total xanthone concentration in root cultures of *H. perforatum* not subjected to any treatment (control) or treated with Cd or As. The numbers next to the bars represent the exact xanthone content

(mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Different letters represent significant differences between samples ($p \leq 0.05$).

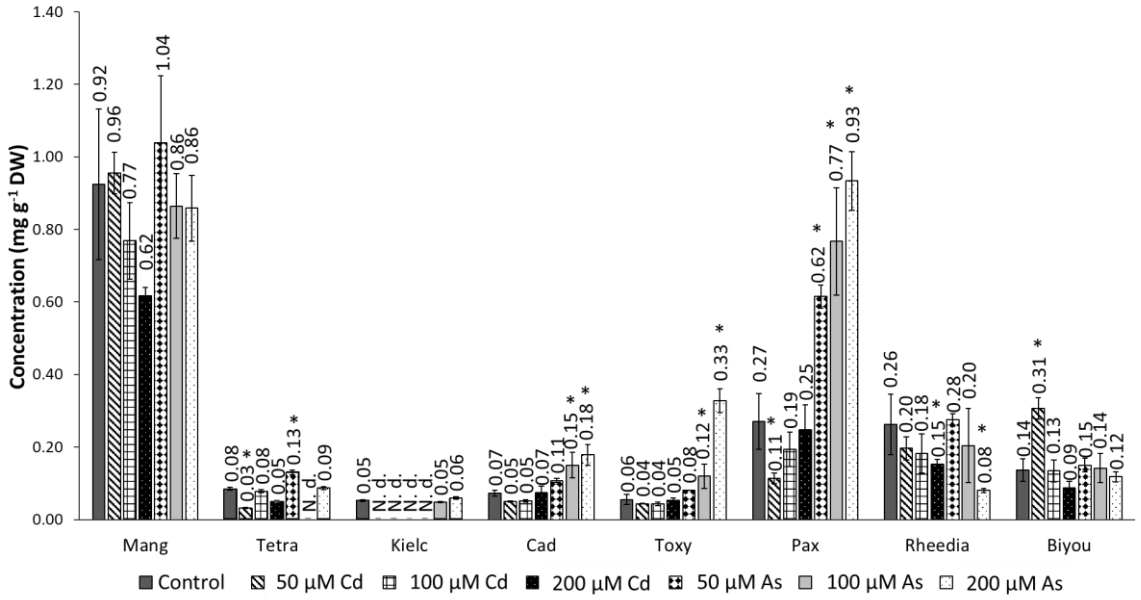

Fig. 3 Xanthone content in *H. perforatum* root cultures not subjected to any treatment (control) or treated with Cd or As. The numbers next to the bars represent the exact xanthone content (mg g^{-1} DW). Results are means (±SD) of three independent biological replicates. Asterisks indicate that the differences between roots and control roots are statistically significant ($p < 0.05$). Mang: mangiferin; Tetra: 1,3,5,6-tetrahydroxyxanthone and 1,3,6,7-tetrahydroxyxanthone; Kielc: kielcorin; Cad: cadensin G; Toxy: toxyloxanthone; Pax: paxanthone; Rheedia: 5-O-methyl-2-deprenylrheediaxanthone; Biyou: biyouxanthone; N.d.: non-detected.

Discussion

The increasing presence of toxic metals and metalloids in the soil due to pollution caused by anthropogenic factors and their consequent absorption by plants, can lead to food chain contamination and to potential poisoning of animals and humans (Su 2014). In *Arabidopsis thaliana* and *Oryza sativa* it has been shown that both cadmium (Cd) and arsenic (As) alter both biosynthesis and transport of auxins (Ronzan et al. 2018; Fattorini et al. 2017). The impact of Cd on secondary metabolism was demonstrated in several species (Zheng and Wu 2004; Rai et al. 2005; Ahmad et al. 2016), while little information is available regarding the relationship between Cd and plant secondary metabolism. Several papers are available on the effect of various toxic metals on *Hypericum perforatum*. It was reported the

accumulation of toxic metals in St. John's wort aerial organs such as chromium, iron, nickel, copper, lead, manganese and zinc (often in very high amounts) and their effect on several parameters such as growth and secondary metabolite production (Murch et al. 2003; Ayan et al. 2006; Obratov-Petković et al. 2008) and the Cd hyperaccumulation (Radanovic et al. 2002; Pavlova et al. 2015).

The purpose of this work was to study the effect of Cd and As on root cultures of *H. perforatum* in terms of xanthone biosynthesis and auxin metabolism to evaluate the root response to toxic metals.

The results obtained showed that xanthone levels were significantly increased in response to 200 mM As. This result emphasizes the non-specificity of plant stress responses compared to animals. Either biotic and abiotic stress can cause the same effect since the first step of both involves ROS production. The studies about growth and morphological alteration of the entire plant should be conducted since was established that As alter these parameters in other species (Rahman et al. 2007; Mokgalaka-Matlala et al. 2008). Contrarily, Cd induced a decrease in xanthone content compared to control (Fig. 2). This result could be read as a consequence of the fact that *H. perforatum* is an hyperaccumulator of Cd (as already reported by Pavlova et al. 2015). It could show lower suffering signs and responses, and a higher resistance to Cd toxicity; although a lower concentration than control suggest a xanthone consumption, probably due to their scavenging effect.

Conclusions

In the present study, the effect of cadmium (Cd) and arsenic (As) was evaluated on St. John's wort root cultures. The results showed that As had a significant effect in stimulating the biosynthesis of xanthones while samples treated with Cd showed a decrement in xanthone production compared to control.

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5. Conclusions

In the present work the effect of biotic and abiotic stress on *Hypericum perforatum* roots was evaluated, administering chitosan oligosaccharides (COS), methyl jasmonate (MeJA), salicylic acid (SA), hydrogen peroxide (H_2O_2), cadmium (Cd) and arsenic (As). This project had both applicative and basic purposes: biotic elicitors may be used in order to stimulate

secondary bioactive metabolite biosynthesis for drug production and to elucidate the influence of shoot/root interaction on elicitor perception in *H. perforatum*; the treatment with toxic metals could help in understanding the processes that occurs when *H. perforatum* grows on polluted soils.

Regarding biotic elicitors, COS allow to discriminate the effect of the elicitor from those of the solvent, being soluble in neutral water, so they are more useful elicitors for basic research than chitosan that requires acidulated water to be solved; they are effective elicitors in enhancing xanthone production in root cultures, with a lower cytotoxic effect on plant cells (than chitosan); xanthone production increased with increasing COS concentration with a maximum production in samples treated with 400 mg L^{-1} COS. Moreover, extracts obtained from COS-elicited roots showed a high antifungal activity against *Candida albicans* and dermatophytes, and the antifungal activity increased with increasing xanthone concentration. COS affected also VOCs biosynthesis in *H. perforatum* root cultures. Regarding other biotic elicitors, MeJA was the most effective elicitor in inducing xanthone biosynthesis in plant roots, while it had no effect on root cultures, the presence of the shoot seems to be necessary for MeJA perception. When used in combination, COS – MeJA and COS – SA appeared to be antagonists in inducing xanthone biosynthesis both in root cultures and plant roots. Contrarily, H_2O_2 enhanced COS effect, showing an additive activity both in root cultures and plant roots.

The experiments with toxic metals showed that the treatment with As significantly enhance xanthone production, contrarily, Cd induced a decrease in xanthone content compared to control, probably because *H. perforatum* is a Cd hyperaccumulator as reported in literature. Bioreactor cultivation tests of *H. perforatum* roots will be necessary in the future in order to obtain a massive xanthone production for pharmaceutical use. Regarding toxic metals, further morphoanatomical, histochemical and biochemical investigations are required to clarify their effect on *H. perforatum* physiology; in the last six months a collaboration with Leibniz Institute of Plant Biochemistry (Halle, Germany) has started in order to evaluate the effect toxic metals on the whole spectrum of endogenous phytohormones.

6. Papers, congresses (presentation or poster) and other activities

Papers

Published

Badiali C, De Angelis G, Simonetti G, Brasili E, de Castro Tobaruela E, Purgatto E, Yin H, Valletta A, Pasqua G (2018). Chitosan oligosaccharides affect xanthone and VOC biosynthesis in Hypericum perforatum root cultures and enhance the antifungal activity of root extracts. Plant Cell Reports https://doi.org/10.1007/s00299-018-2317-2.

Valletta A, De Angelis G, Badiali C, Brasili E, Miccheli A, Di Cocco ME, Pasqua G (2016). Acetic acid acts as elicitor exerting a chitosan-like effect on xanthone biosynthesis in Hypericum perforatum L. root cultures. Plant Cell Reports 35:1009-20.

In preparation

Badiali C, Brasili E, Iozia LM, Petruccelli V, Caparra MV, Di Giovenale A, Pasqua G, Valletta A. Response of Hypericum perforatum root cultures and in vitro-grown plantlets to chitosan oligosaccharides (COS), methyljasmonate (MeJA), salicylic acid (SA) and hydrogen peroxide (H_2O_2).

Badiali C et al. Effect of cadmium and arsenic on xanthone production in Hypericum perforatum root cultures

Congresses

Pasqua G, Badiali C, Brasili E, De Angelis G, Simonetti G, de Castro Tobaruela E, Purgatto E, Yin H, Valletta A (2018). Water-soluble chitosan oligosaccharides (COS) affect xanthone and volatile organic compound content, as well as antifungal activity against human pathogenic fungi of extracts obtained from Hypericum perforatum root cultures. In 113° Congresso della Società Botanica Italiana, V INTERNATIONAL PLANT SCIENCE CONFERENCE (IPSC) Fisciano (Salerno), 12-15 September 2018. ABSTRACTS KEYNOTE LECTURES, COMMUNICATIONS, POSTERS - ISBN 978-88-85915-22-0, p. 104, vol.1

Badiali C (**speaker**), Brasili E, De Angelis G, Simonetti G, de Castro Tobaruela E, Purgatto E, Yin H, Valletta A, Pasqua G (2018). Effect of chitooligosaccharides on xanthone and

volatile compound biosynthesis in Hypericum perforatum L. root cultures. In Riunione scientifica congiunta (a cura di B. Ruffoni, A. Giovannini e M. Savona), Sanremo, 13-15 June 2018

Badiali C (**speaker**), Valletta A, De Angelis Giulia, Pasqua G (2017). Effect of chitosan oligosaccharides and other biotic elicitors on root cultures and in vitro grown plantlets systems of Hypericum perforatum L. In 112° Congresso della Società Botanica Italiana, IV INTERNATIONAL PLANT SCIENCE CONFERENCE (IPSC) Parma, 20 - 23 September 2017. ABSTRACTS KEYNOTE LECTURES, COMMUNICATIONS, POSTERS - ISBN: 978-88-85915, p. 51, vol. 1

Valletta A, De Angelis G, Badiali C, Brasili E, Miccheli A, Pasqua G (2017). Acetic acid acts as an elicitor exerting a chitosan-like effect on xanthone biosynthesis in Hypericum perforatum L. root cultures. In: Riunione annuale dei gruppi di lavoro SBI - Biologia Cellulare e molecolare & Biotecnologie e Differenziamento. Milano 14-16 June 2017. p. 31, vol. 1

Valletta A, De Angelis G, Badiali C, Simonetti G, Pasqua G (2016). Chitooligosaccharides (cos) enhance xanthone production in Hypericum perforatum root cultures. III International Plant Science Conference (IPSC) Società Botanica Italiana, 111° Congresso. Roma Tor Vergata 21-23 September 2016. ABSTRACTS KEYNOTE LECTURES, COMMUNICATIONS, POSTERS ISBN: 978-88-85915-18-3 p. 125, vol. 1

Valletta A, De Angelis G, Badiali C, Simonetti G, Pasqua G (2016). Water-soluble chitooligosaccharides (COS) elicit xanthone biosynthesis in Hypericum perforatum root cultures. In: Riunione Annulae dei Gruppi di Lavoro SBI - Biologia Cellulare e Molecolare & Biotecnologie e Differenziamento. Amantea (CS), 14-16 June 2016. p. 70, vol. 1

Valletta A, De Angelis G, Badiali C, Simonetti G, Pasqua G (2016). Water-soluble chitooligosaccharides (COS) elicit xanthone biosynthesis in Hypericum perforatum root cultures. In: Riunione Annuale dei Gruppi di Lavoro SBI - Biologia Cellulare e Molecolare & Biotecnologie e Differenziamento. Amantea (CS), 14-16 June 2016. p. 70, vol. 1

Internship

Leibniz Institute of Plant Biochemistry – Halle, Germany (3 months)

Tutoring

Bachelor's and Master's deegree students

Courses

Identifying Metabolite Families from untargeted metabolomics using MetFamily - Leibniz Institute of Plant Biochemistry, (Halle, Germany)

Metabolomics data processing with MS-Dial - Leibniz Institute of Plant Biochemistry (Halle, Germany)

"R" - "Sapienza" Università di Roma

I Corso Microscopia elettronica e confocale in ambito botanico - Università di Modena e Reggio Emilia