Hypericum perforatum Cultures as a Tool to Study Plant Defence Mechanisms Against Anthracnose (*Colletotrichum gloeosporioides*)

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ABSTRACT

To elucidate the defence mechanisms of *Hypericum perforatum* L. against *Colletotrichum gloeosporioides*, we utilized cell suspension cultures. When primed with methyl jasmonate, *H. perforatum* cells showed a double oxidative burst upon *Colletotrichum gloeosporioides* elicitation typical of a hypersensitive response. Phenolic profile of the cells was modified upon various stimuli such as salicylic acid, MeJ and *C. gloeosporioides*. The possible importance of reactive oxygen species production and phenolics (xanthones) as components of defence mechanism of *H. perforatum* against biotic stress is discussed.

1. INTRODUCTION

St. John's wort (*Hypericum perforatum*; HP) is a medicinal plant widely used in folk medicine in several countries. Its properties have been mentioned since ancient times, by physicians like Dioscorides, Galen and Plinio. In the Medieval age, St. John's oil was a very famous unguent utilised as a remedy for several ailments. The earlier use of St. John's wort was mainly based on traditional knowledge. In the past decades, systematic approaches in phytochemical, pharmacological and clinical studies gave a better knowledge of the medicinal properties of this plant. Recently, the potential of HP extracts or compounds to act as antiviral (Miskovsky 2002), antitumour (Schempp *et al*. 2002), neuroprotective (Silva *et al*. 2005), and antioxidant (Silva *et al*. 2004) agents have been investigated. Hypericin has been considered as a promissory compound for the photodynamic therapy of cancer (Agostinis *et al*. 2002, Blank *et al*. 2004).

Nowadays, the utilization of HP extracts for the treatment of depression has become prominent and some recent reviews focusing this issue can be pointed out (Erdelmeyer *et al*. 2000, Di Carlo *et al*. 2001, Butterweck 2003). Several studies have successfully demonstrated the clinical effectiveness of HP preparations in properly controlled clinical trials (Trautmann-Sponsela and Dienel 2004, Szegedi *et al*. 2005). As a consequence, the demand for St. John's wort derived pharmaceuticals and dietary supplements have drastically increased in recent years (Wills *et al*. 2000). Presently, St. John's wort is one of the best-selling herbal medicines worldwide with an important share in the phyto-pharmaceutical and OTC market of Europe and USA.

The commercially available HP derived products include sophisticated phyto-pharmaceuticals, tinctures, teas, juices and oily macerates. Also, several brands of common food products, such as beverages and yoghurts, include St John's wort extracts as additives. A major dilemma faced by consumers, manufacturers, and regulators is the high degree of variability in the product quality. Efforts are being made by the phyto-pharmaceutical industry in order to improve the quality and constancy of the supply of *Hypericum* products. Nowadays, most of the commercial products are derived from selected HP accessions cultivated in the field instead of wild plant collections as raw material. Today, HP cultivation covers several hundred hectares in Europe (Gaudin *et al*. 2003).

Anthracnose caused by the fungus *Colletotrichum gloeosporioides* (CG) is a major problem in field cultivation of HP. This fungus is responsible for significant losses in HP plantations by lowering the yield and eventually modifying chemical composition of the biomass produced. Extensive efforts have been taken to obtain HP plants resistant to anthracnose but, until now, this goal has not been achieved. Understanding the biochemical and molecular basis of resistance is a prerequisite to obtain disease-resistant varieties, when natural resistance is inadequate. Nevertheless, little is known about the defence responses of HP against pathogen attack. Recently, differential accumulation of hyperforin and hypericin in HP plantlets was observed as a response to CG elicitation (Gibson and Sirvent 2002).

Abbreviations: CG, *Colletotrichum gloeosporioides*; **HR**, hypersensitive response; **HP**, *Hypericum perforatum*; **MeJ**, methyl jasmonate; **ROS**, reactive oxygen species; **SA**, salicylic acid; **1,3,6,7-xanthone**, xanthone with the 1,3,6,7 oxygenation pattern

Plant cell cultures are in general useful tools to study several aspects of plant biology, including plant defence. They have been utilized, with success, as models to study biochemical changes related to plant defence responses against pathogens (Hagemeir *et al*. 1999, Conrath *et al*. 2002, Hahlbrock *et al*. 2003). These systems offer some advantages, compared to the *in vivo* plant-pathogen interaction, which includes the simplicity of manipulation and a precise cause-effect determination. The use of cell cultures has also been of great help for the identification of the signaling pathways and timing of the events activated during plant defense responses. This is because they are uniform systems in which identical cells are equally exposed to a certain stimulus and, thus, simultaneously activate their response (De Gara *et al*. 2003). Taking this in consideration, the use of *in vitro* cultures to study the defence response of HP against CG could give new insights for disease control.

2. MODULATION OF THE PRODUCTION OF REACTIVE OXYGEN SPECIES (ROS) IN *H. PERFORATUM* CELLS

In normal conditions, ROS are routinely generated at low levels in non-stressed plant cells in chloroplasts and mitochondria, and also by cytoplasmic, membrane bound or exocellular enzymes involved in redox reactions (Wojtaszek 1997). The majority of cells have also acquired the protective mechanisms to maintain the lowest possible levels of ROS inside the cell. In some cases, however, especially under stress conditions such as pathogen attack, these protective mechanisms are overdriven by the rapid, transient production of a huge amount of ROS, named the 'oxidative burst' (Lamb and Dixon 1997, Mackerness *et al*. 2001, Scandalios 2002, Torres *et al*. 2002).

The purpose of ROS production in plants includes: i) direct killing of pathogens, by the generation of extremely reactive radicals like the hydroxyl radical (De Gara *et al*. 2003); ii) involvement in cell wall stiffening by catalysing intra- and inter-molecular cross-links between structural components of cell walls and lignin polymerisation (Ros-Barceló 1997); iii) promotion of the programmed cell death (Scandalios 2002) and iv) to serve as signalling molecule to activate further plant defence (Tenhaken *et al*. 1995).

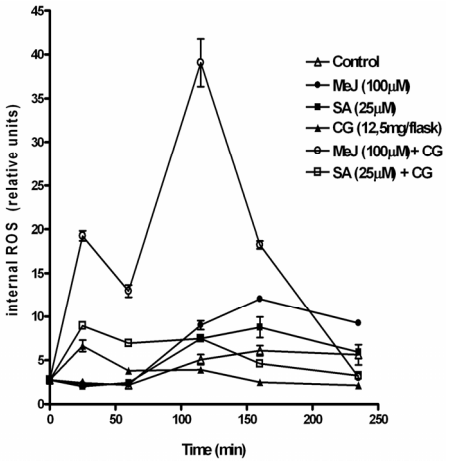

Fig. 1 ROS production in response to various stimuli. Note the typical double ROS burst in HP cells elicited with CG after priming with MeJ.

In most incompatible responses the rapid induction of ROS determines unfavourable conditions for pathogen growth. These may result in the death of small number of plant cells at the site of infection, known as hypersensitive response designed to impair pathogen spread. In a series of experiments, we studied the ROS production in HP cells in response to various stimuli (Conceição *et al*. 2003, Ribeiro and Dias 2004). HP cells were challenged with autoclaved CG biomass with or without prior treatment (24 h) of cultures with 25 µM of salicylic acid (SA) or 100 µM methyl jasmonate (MeJ). Individual effects of SA and MeJ on ROS production were also checked. Quantity of ROS produced internally by HP cells was measured by a fluorescent probe (2',7'- dichlorofluorescein diacetate) during the first 5 hours of culture, following the addition of elicitors. The ROS production in response to various treatments is shown in the **Fig. 1**. An initial ROS burst was observed in the HP cells after 25 min of elicitation with CG. This initial ROS burst was more intense when the HP cultures were primed with SA or MeJ before exposing to the CG extract. However, MeJ-primed cells produced significantly more ROS than those pre-treated with SA. A very intense second ROS burst (125 min after treatment) due to CG elicitation was observed only in MeJ-primed HP cells. This reaction is typical of a hypersensitive response, characteristic of an incompatible plant-pathogen interaction.

The mechanism of action of SA and MeJ (or more general, jasmonates) is still in debate (Felton and Korth 2000). These two compounds seem to act independently *via* antagonising pathways giving rise to various plant responses. Similarly, in the present study also the ROS production upon CG elicitation was also differentially mediated by MeJ and SA. Our results also explain that the hypersensitive response in HP cells is MeJ- mediated.

3. MODULATION OF THE PRODUCTION OF PHENOLIC COMPOUNDS IN *H. PERFORATUM* CELLS

In the presence of a pathogen, plants develop a vast array of secondary metabolic defence responses sequentially activated in a complex multi-component network including the production of several phenolics (Dixon and Paiva 1995, Dixon 2001, Tan *et al*. 2004). The phenolics produced might contribute to disease resistance in plants as new inducible low molecular-weight compounds, called 'phytoalexins'. These compounds are post-infectional, i.e. although they might already be present at low concentrations in the plant, they rapidly accumulate upon pathogen attack.

Several studies concerning the plant defence response against *Colletotrichum* sp. infection have been done. *Colletotrichum lagenarium* caused enhanced incorporation of cell wall-associated phenolics in cucumber (Daen and Kuc 1987). Epicatechin was involved in the resistance of unripe avocado fruits to CG (Wattad *et al*. 1994). Reduction in the phenolic content of leaves of water lilies was associated with a higher disease severity caused by *C. nymphaeae* (Vergeer and van der Velde 1997).

The possible relevance of phenolics in the defence mechanisms of HP against CG was evaluated (Conceição *et al*. 2003 2004 2005). HP cell cultures were primed and elicited as described in the previous section. Soluble phenolics were analysed by HPLC-DAD and HPLC-DAD-MS/MS during the course of interactions, as described elsewhere (Dias *et al*. 1999, Silva *et al*. 2005).

In normal conditions, the major groups of phenolic compounds produced by HP cell suspension cultures were xanthones with the oxygenation patern 1,3,6,7 (1,3,6,7-xanthones) and quercetin derivatives (**Fig. 2A**), as already reported elsewhere (Dias *et al*. 2001, Dias 2003). We observed significant changes in the phenolic profile of HP cells upon various stimuli. When treated with MeJ, HP cells produced several new compounds that were not detected in the control cells (**Fig. 2B**). Those compounds were a new set of flavonoids, the flavones, and were identified as luteolin and apigenin derivatives (Conceição *et al*. 2005). Luteolin and other related flavones were already identified in HP cells (Dias *et al.* 1998). However, these new compounds were not detected in cells exposed only to the CG (**Fig. 2C**). CG elicitation induced the production of many new 1,3,6,7-xanthone derivatives in both cells primed and un-primed with MeJ (**Figs. 2C, 2D**). A similar occurrence was observed with cells primed with SA and then elicited with CG-elicitor.

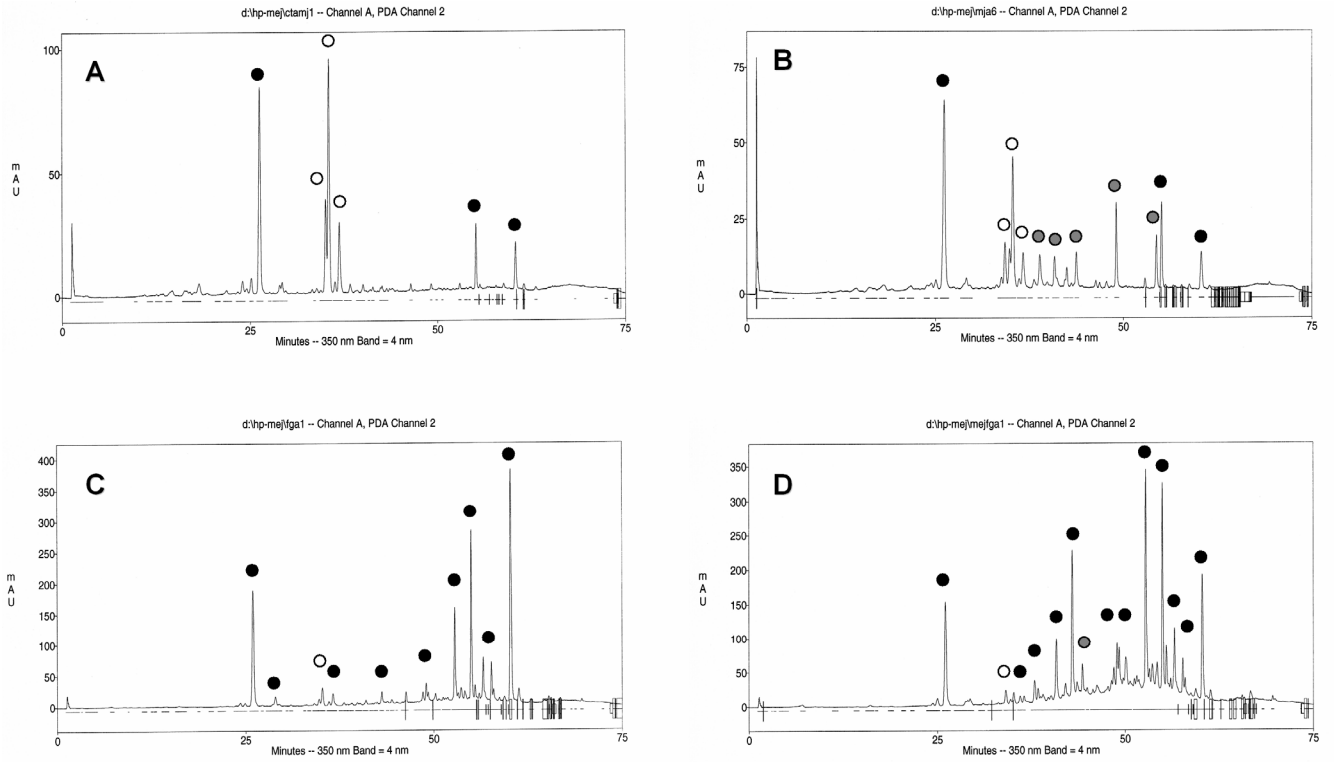

Fig. 2 HPLC-DAD chromatograms of methanolic extracts from *H. perforatum* cells, on the 7th day of culture. (**A**) Control samples, (**B**) MeJ-treated cultures, (**C**) cultures elicited with *C. gloeosporioides* elicitor and (**D**) cultures primed with MeJ, prior to *C. gloeosporioides* elicitation. Compounds: 1,3,6,7-xanthone derivatives (●); flavonols- quercetin derivatives (○) and flavones- luteolin and apigenin derivatives (●).

The amounts of various phenolic compounds were also changed due to priming and elicitation treatments (**Fig. 3**). HP cells elicited with CG showed seven times higher xanthone accumulation than the control**.** This xanthone accumulation increased further when the cells were primed with SA or, specially, with MeJ (12-fold increase), before CG elicitation. Addition of MeJ alone to HP cultures did not show any effect on xanthone accumulation. However, SA induced a small but significant increase in xanthone accumulation. On the other hand, flavonoid accumulation almost ceased after CG elicitation in both primed and un-primed cells.

Xanthone accumulation changed differentially throughout the growth of HP cells after priming and elicitation. In the cell suspensions elicited with MeJ, xanthone accumulation remained similar to control throughout the experimental period (**Fig. 4**). However, cells elicited with SA showed a significant increase in xanthone production with culture period (P<0.05). Xanthone level of HP cells primed with MeJ reached the maximum in 24 h after the addition of CG elicitor (**Fig. 4**) and that level persisted continuously for two days. Cell suspensions treated with fungal elicitor with or without SA priming attained the highest xanthone accumulation only after 72 h of elicitor addition and thereafter, the amount started to decline gradually. Cells elicited with the CG-elicitor reached the typical amounts that were found in the control on day 12. However, cells primed with MeJ or SA prior to the addition of CG-elicitor have retained a significant higher xanthone level than other treatments even after 12 days (**Fig. 4**).

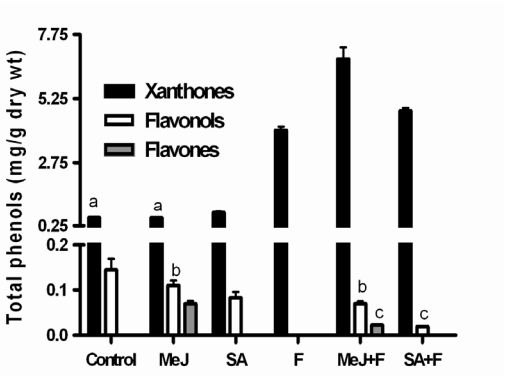

Fig. 3 Total phenols produced at the 7th day of culture, 24h after fungal elicitor addition to the cell cultures of *H. perforatum*. Cultures were treated with MeJ (100 µM), SA (25µM), or CG elicitor (F). The bars indicated as MeJ+F and SA+F correspond to cells primed with MeJ (100 µM) and SA (25µM), respectively, before the addition of the fungal elicitor. Results are means (±SD) of six independent replicates from two independent experiences. All the values are statistically different (P<0.05) except those indicated with the same letter.

Flavonols accumulation significantly decreased after the addition of MeJ and SA in HP cells on day 3 and thereafter (**Fig. 4**). Addition of CG elicitor resulted in the rapid disappearance of flavonols within three days in both primed and un-primed cells. It is interesting to mention that this disappearance coincided with the onset of xanthone production (**Fig. 4**).

The new flavones which were induced by MeJ (**Fig. 2B**) accumulated during the first 48 h and subsequently declined in HP cells (**Fig. 4**). Addition of the CG elicitor to those cells primed with MeJ resulted in a rapid fall of flavone level until they could no longer be detected after 72 h.

The several fold increase in xanthone accumulation could be the reason why flavonoids (flavonols + flavones) became undetectable after elicitation with CG (**Figs. 2C, 2D, 4**). It is known that xanthones and flavonoids are biosynthetically related compounds, sharing a pool of precursors (Shröeder 1997, Dias 2003, Liu *et al*. 2003). As a response to CG, those precursors might have been shifted for xanthone biosynthesis in detriment to the flavonoid pathway resulting in a too low flavonol and/or flavone production to be detected by HPLC.

Beerhues and Berger (1995) already observed an increase in the accumulation of new xanthones in the cultures of *Centaurium* sp. upon elicitation with yeast. Xanthones are known for their pharmacological activities (Hostettmann and Hostettmann 1989) such as antibacterial and anti-fungal (Braz-Filho 1999). In addition, xanthones produced by *Hypericum androsaemum* cell cultures have shown to inhibit the growth of *Candida utillis* and *Saccharomyces cerevisae* (Dias 2003). Moreover, many xanthones accumulated after elicitation have a non-polar nature, which renders higher antimicrobial activity to the compounds. Therefore, the increase in xanthone accumulation observed in HP cells can be described as a defence response triggered by some of the components present in the fungal elicitor. Fungal components such as proteins,

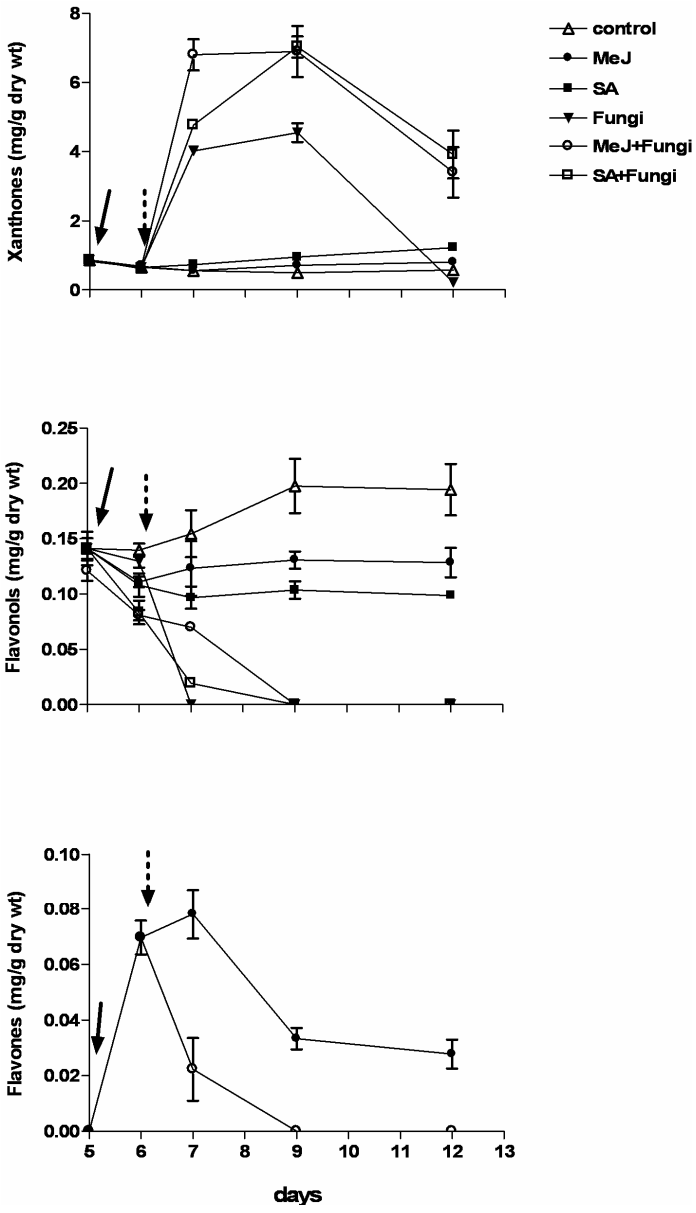

Fig. 4 Accumulation of phenolic compounds by cell suspension cultures of *H. perforatum,* during culture growth period, treated with MeJ (100 µM), SA (25 µM), or CG elicitor (F). The samples indicated, as MeJ + F and SA + F were cells primed with MeJ (100 µM) and SA (25 µM), respectively, before the addition of the fungal elicitor. Results are means (±SD) of six independent replicates from two independent experiences. The straight arrow indicates the addition of SA or MeJ, and the dotted arrow indicates addition of fungal elicitor.

glycoproteins or oligosaccharides can trigger the defence mechanisms in plants (Dmitriev 2003).

Even though plant defence can be triggered by local recognition of pathogens, more effective responses include signalling pathways such as the Acquired Systemic Response depend on SA signalling (Dempsey *et al*. 1999) and the Induced Systemic Resistance, depend on jasmonic acid (JA) signalling (Feys and Parker 2000). SA, JA and its derivatives like MeJ have been used as inducers of plants defence and were found to stimulate their secondary metabolism (Thomma *et al*. 2000, Hahlbrock *et al*. 2003).

Xanthone accumulation in HP cells is a direct response to CG elicitation. In the present case, both SA and MeJ were able to induce xanthone accumulation. SA and jasmonate (or MeJ) seem to act by independent and occasionally antagonistic pathways, giving different plant responses (Felton and Korth 2000). In HP cells, SA was able to increment the production of xanthones by itself, whereas MeJ alone did not interfere significantly with xanthone biosynthesis but resulted in a selective accumulation of flavones. The function of those new flavones on HP-CG interactions is not clear and remains to be elucidated.

Vivanco *et al*. (2002) observed an increase in hypericin production of HP cultures, when treated with JA and not with SA or a pathogen extract. On the contrary, the elicitation of HP plantlets with SA, MeJ and CG resulted in a differential accumulation of hyperforin and hypericin depending on the treatments (Gibson and Sirvent 2002). In our case, we did not observe the accumulation of either hypericin or hyperforin in HP cultures after elicitation with CG in agreement with our previous report (Dias 2003). This could be due to the fact that compounds like hypericins are accumulated in specialized tissues (glands) and not in cells of an undifferentiated state.

4. CONCLUSION

The ROS and xanthone production in HP cells due to CG elicitation are differentially mediated by SA and MeJ (**Fig. 5**). The double ROS burst due to CG elicitation after priming with MeJ is typical of a hypersensitive response, characteristic of an incompatible plant-pathogen interaction. It seems that MeJ acts as the signalling molecule and is an important component in HP plant defence. The accumulation of xanthones in HP cultures after CG elicitation strongly indicates that these compounds could act as phytoalexins. Since ROS and secondary metabolites (phenolics) can be effectively studied in cell suspension cultures of HP, they could serve as a suitable model to study HP-CG interactions.

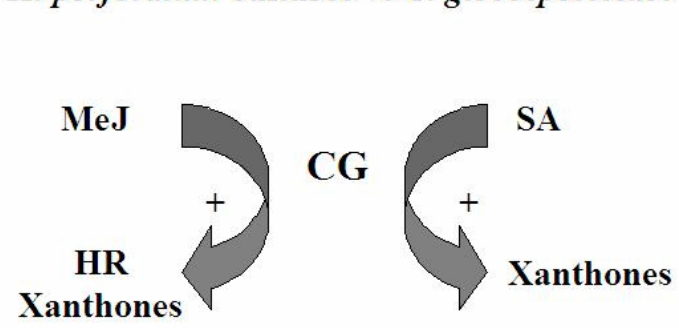

Fig. 5 Hypothetical model of HP-CG interactions mediated by SA and MeJ; HR-hypersensitive response.

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