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In vitro biological activity of C-glycosylflavonoids.

SUMMARY

C-glycosyl-flavones are toxic *in vitro* for *Verticillium albo atrum* and *Phytophthora parasitica*. Equivalents concentrations of these compounds cause similar cytological alterations of both parasites, inhibit their growth and asexual reproduction. These phenolics do not seem to be detoxified during incubation. They are better inhibitors of pectic transeliminases than of pectic hydrolases.

INTRODUCTION

In several interactions between fungal or bacterial pathogens and higher plants, phenolics are detected among substances which could contribute to the biochemical reaction of the hosts. Some of these compounds are pre-infectional constituents. In other cases they are biosynthesized in tissues after infection. These compounds are frequently benzoic or cinnamic acids, flavonoids, isoflavonoids or their derivatives.

Among the flavonoids, C-glycosylflavonoids are characterized by a stable carbon-carbon bond between the phenolic nucleus and the glycosidic moiety. They are rather widely distributed in the plant kingdom: Gramineae, Caryophyllaceae, Compositae, Cucurbitaceae, Euphorbiaceae, Leguminosae, Palmae, Rutaceae, Sterculiaceae, Verbenaceae.... In these families, except Leguminosae,

the chemical factors which contribute to the mechanisms of resistance are partially identified or unknown.

Our aim was to determine the biological activity of C-glycosyl--flavones on the vegetative cycle of two fungi - a Syphomycete, Phytophthora parasitica and a Septomycete, Verticillium albo atrum - and on the activity of their pectinolytic enzymes.

MATERIALS AND METHODS

- C-glycosyl-flavones

The compound used for this study are shown in Figure 1. They were vitexin (8-C-β-D-glucopyranosylapigenin) extracted from Vitex lucens, iso-orientin (6-C-β-D-glucopyranosylluteolin) from Aspalathus acuminatus, molludistin (8-C-α-L-arabinopyranosylgenkwanin) and iso-swertisin (8-C-β--D-glucopyranosylgenkwanin) from Mollugo distica, schaftoside (6-C-β-D-gluco-pyranosyl 8-C-α-Larabinopyranosylapigenin) from Silene schafta. The most distinctive features of these compounds concerned the nature, the position and the number of sugars.

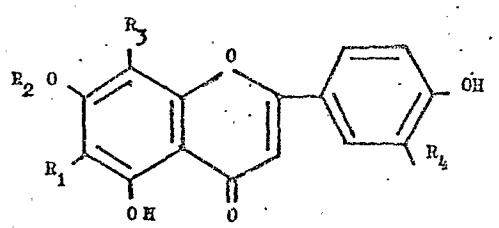

Fig. 1

Fig. 1. – Vitexine : $R_1 = R_2 = R_4 = H$. R_3 = β-D-glucopyranosyl; Iso-orientine : R_1 = β-D-glucopyranosyl. $R_2 = R_3 = H$, $R_4 = OH$; Molludistine : $R_1 = R_4 = H$, $R_2 = CH_3$, R_3 = α-L-arabinopyranosyl; Iso-swertisine : $R_1 = R_4 = H$. $R_2 = CH_3$, R_3 = β-D-glucopyranosyl; Schaftoside : $R_2 = R_4 = H$. R_1 = β-D-glucopyranosyl, R_3 = α-L-arabinopyranosyl.

- pathogens and bioassays

The strains of P. parasitica and V. albo atrum were isolated from Tomato. A liquid medium containing mineral salts, glucose and thiamine was used for tests on closed concavity slides or in vials. Ethanolic solutions of

the compounds were used so that final concentrations ranged from 5×10^{-6} to 3×10^{-5} .Plugs used as inoculum,100 µ to 5 mm,were picked on 4-7 or 15 days old pea agar cultures.

Incubations were carried out from 4 to 45 days at 30°C.Mycelial modifications were checked by light microscopy on concavity slides.The cultures were then transferred in tubes containing pea agar medium to determine their ability to recovery.

After several incubations,C-glycosyl-flavones were extracted from the solutions and controlled by TLC on silica gel with ethyl acetate-methanol-water (63:12:9).Dosages were carried out by UV spectrometry.

- Pectinolytic enzymes

They were endo pectin hydrolases -endo PMG- from commercial extracts (Pectinases Fluka and Sigma) and endo pectin transeliminases -endo PTE- from P. parasitica cultures and pectinase Fluka.The degradation rate of 2 substrates respectively methylated at 30% and 75% (Unipectine SA) by an endo PMG in 0.1 M citrate buffer (pH = 4.5 - 5) was determined after thiobarbi--turic acid (TBA) treatment,spectrometrically from 490 to 530 nm .
The activity of endo PTE on the 75% esterified pectin was measured in 0.1 M tris H Cl buffer (pH = 7.2 - 8) spectrometrically in UV - 226 / 238 nm - or in visible after TBA treatment - 530 / 560 nm - .The inhibitors were incorporated so that the inhibitor/enzyme concentration ratio was ranged from 1/3 to 1/1 .

RESULTS

- inhibition of fungi

C-glycosyl-flavones caused in the hyphae of both fungi alterations of the wall structures and of the cytoplasmic content.Growth and ramifi--cation,chlamydospore formation and asexual reproduction were also altered.

In cultures of P. parasitica these compounds at concentration of 5×10^{-6} induced formation of vesicular,torulous and often gathered hyphae. Walls were very refringent and showed irregular thickenings toward the apex. Pseudo-septa are formed in cytoplasm which was progressively vacuolized.The formation of sporocystes was inhibited and those introduced with the inoculum shrivelled slowly.Concentrations of 10^{-5} caused a generalised cytoplasmic lysis which also affect the content of most chlamydospores. A similar

toxicity was observed in cultures of V. albo atrum .

The growth of both fungi decreased of about 1/3 to 1/2 compared to the control for a concentration of 5×10^{-6}. Inhibition set up slowly and persisted during the whole contact period with the inhibitors. At a concentration of 10^{-5}, the growth inhibition occured after a few hours. The size of the plugs of inoculum and the transfer of nutritive reserves did not modify the sensivity of pathogens.

Lethal doses were dependent on the vegetative stage of the P. parasitica and V. albo atrum cultures from which plugs were taken out for bioassays. In growing hyphae, lethal doses approached 2×10^{-5}. Nevertheless, when cultures had differentiated chlamydospores, lethal doses were larger and probably depended on the permeability of the chlamydospores walls.

Verifications carried out at the end of several incubations, even in case of moderated mycelial growth, did not allow to determine any detoxification of the compounds.

- Inhibition of pectinolytic enzymes

+ Endo PMG :

The inhibition of enzymes hydrolysing the 75% or the 30% methylated substrates was similar. Four compounds appeared to be weakly inhibitors : vitexin, iso-orientin, molludistin and iso-swertisin. They decreased the enzymic activity by 18, 20 and 25% in 1/3, 1/2 and 1/1 effector: enzyme ratios respectively. Schaftoside was more effective; in the same conditions, the enzymic activity decreased by 20, 35 and 50% respectively. The inhibition of the endo PMG was not competitive for the substrate as shown by Lineweaver-Burk plots in figure 2 .

+ Endo PTE :

The efficiency of inhibition of endo PTE from Pectinase Fluka or synthesized by P. parasitica was intensive and quite independent from structural differences between inhibitors. Thus, the activity of endo PTE from Pectinase Fluka decreased by 85 and 95% in 1/2 and 1/1 effector: enzyme ratios respectively. In this case, the inset of inhibition was quicker than with endo PMG. The readings made in UV revealed that endo PTE activity was stopped after 3 to 6 hours of incubation. The inhibition of endo PTE was not competitive for the substrate and did not seem cumulative.

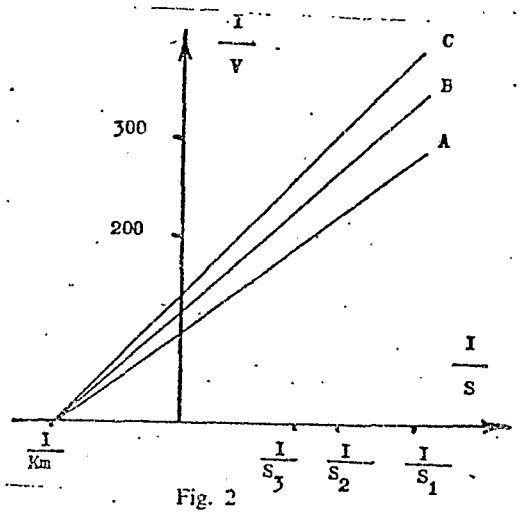

Fig. 2

Fig. 2 - Curves of Lineweaver-Burk for activity of endo PMG and it inhibition by schaftoside : A=reference, B, C=inhibition with effector:enzyme ratios 2/3 and 1/1 .

DISCUSSION

In our experiments, C-glycosyl-flavones were equally fungitoxic for both pathogens in spite of cytological and structural differences between hyphae (mainly the lack of sterols in the walls of P. parasitica hyphae). The fungistatic doses of C-glycosyl-flavones, expressed in concentrations of apigenin, luteolin or genkwanin, appeared comparable to those previously reported for the phenolic compounds extracted from Gossypium (Ravisé and Trique 1972) and Nicotiana (Ravisé and Tanguy 1973). Nevertheless, the C-C bond between the carbohydrate moiety and the phenolic nucleus may modify the biological properties of the latter. Particularly, the hyphal walls of fungi could be as permeable to these compounds as to the corresponding sugars, while the hydrolytic enzymes of fungi would be unable to break the C-C bond. According to our results, C-glycosyl-flavones seem more toxic than the corresponding glycosides, mainly those of isoflavones from soybean (Naim and coll. 1974) .

Chromatographic and spectrophotometric verifications showed that alterations noticed for the parasites did not result from a preliminary oxidation of hydroxyls into quinones. These results support our former studies concerning the toxicity for P. parasitica of coumarins, homo-isoflavanones, isoflavonoids and coumestans or their methyl ethers (Ravisé and Kirkiacharian 1976 - 1978) .

C-glycosyl-flavones do not seem to be detoxified during the incubation with both fungi, even in case of moderate mycelial growth. This stability, if confirmed with other pathogens, would lead to distinguish these compounds from isoflavonoids, pterocarpans and sesquiterpenoids which are

detoxified by several microorganisms (Michele Heath and Verna Higgins 1973, van den Heuvel and van Etten 1973, Stoessl and coll. 1973, Ingham 1976....) .

The incidence of C-glycosyl-flavones on the activity of pectino--lytic enzymes considerably contrasts in the case of endo PMG and endo PTE, the latter being strongly inhibited. For each group, the inhibition does not relate with the origin of enzymes nor with their probable structural differences. The binding of flavones with one or two molecules of sugar and consecutive steric conformation do not hinder the enzymatic inhibition. Moreover, as we determine formerly for isoflavonoids, coumestans and coumarins molecular substitutions on the compounds modify the function of enzymic effector (previous ref.) .

C-glycosyl-flavones are synthesized in relative large amounts in tissues of Gramineae - mainly Oats, Wheat and Barly - during the first stages of growth. These compounds are also known in tropical cultures : Hevea, Citrus, COCOA.... For these crops, further investigations might study in vivo contribution of C-glycosyl-flavones to the inhibition of pathogens, mainly of Phytophthora sp. and their pectic transeliminases.