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(E)-p-coumaroyl-1-O-β-D-glucopyranoside accumulation in roots of *Plantago lanceolata* cultures

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Summary.- Biotransformation of 1 mM *(E)*-cinnamic acid by thirty-day-old whole ribworts (*Plantago lanceolata*) was investigated. After addition of this caffeic acid precursor in medium culture, the root accumulation of the two main caffeic acid glycoside esters (CGEs), plantamoside and verbascoside was compared with *in vitro* ribwort control. No major modification of these two CGEs storage was highlighted. *p*-Coumaroyl-glucose and feruloyl-glucose were neosynthesized and accumulated in the roots of seedlings fed with cinnamic acid. Maximum level of *p*-coumaroyl-glucose was higher than those produced by seventy-five-day-old ribworts previously investigated. Hence, the complete structure of the latter glucosylated acid was determined by both ^{1}H and ^{13}C NMR spectroscopy as *(E)-p*-coumaroyl-1-O-β-D-glucopyranoside.

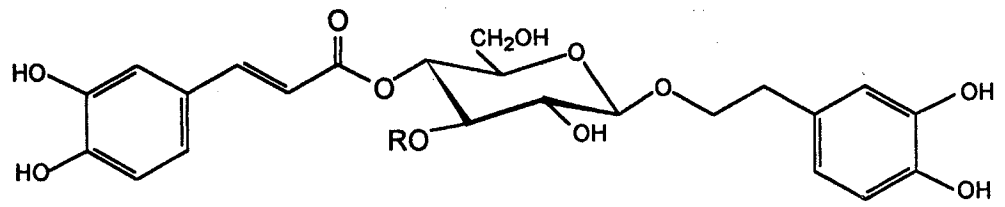
Résumé.- La biotransformation de l'acide *(E)*-cinnamique par des plantules entières de *Plantago lanceolata* âgées de 30 jours a été étudiée. Après addition de ce précurseur de l'acide caféique dans le milieu de culture des plantules, l'accumulation du plantamoside et du verbascoside, principaux esters hétérosidiques de l'acide caféique (EHAC) des racines de *P. lanceolata*, a été comparée à celle de plantules témoins. Aucune modification importante des taux de ces deux EHAC n'a pu être caractérisée dans les racines de *P. lanceolata*, mais la néosynthèse et le stockage de féruloyl-glucose et de *p*-coumaroyl-glucose dans les racines des plantes, cultivées avec l'acide cinnamique, ont été mis en évidence. Le taux maximum de *p*-coumaroyl-glucose accumulé dans les racines de plantules de 30 jours est deux fois plus élevé que celui des racines de plantules de 75 jours. De plus, la détermination structurale complète de ce dérivé glucosylé par spectroscopie en RMN du ^{1}H et du carbone ^{13}C montre qu'il s'agit du *(E)-p*-coumaroyl-1-O-β-D-glucopyranoside.

Key-words : *Plantago lanceolata* - *(E)*-cinnamic acid – plantamoside – verbascoside – biotransformation.

I. INTRODUCTION

In traditional medicine, *Plantago lanceolata* (ribwort), one of the most common *Plantago* species is used for antibacterial, anti-inflammatory, healing, anti-asthmatic and diuretic properties (Rombi, 1992; Valnet, 1992; Leclerc, 1994; Zapata, 1996). Among its several active components, caffeic acid glycoside esters (CGEs) are antibacterial, antifungal, antiviral, antioxidant and selective inhibitors of aldose reductase, 5-lipoxygenase and protein kinase C (Ravn and Brimer, 1988; Herbert *et al.*, 1991; Andary, 1993; Wang *et al.*, 1996). A previous work reported that the two main CGEs of *P. lanceolata* (Fig. 1), plantamoside (P) and verbascoside (V) (Andary *et al.*, 1988) were concentrated in the roots of ribworts with P levels double those of V (Fons *et al.*, 1998). In order to modify ribwort CGEs profile, 75-day-old-seedlings were transferred into MS medium containing 1 mM *(E)*-cinnamic acid. This caffeic acid precursor has been shown to be integrated into the polyphenolic pathway (Molderez *et al.*, 1978; Moriguchi *et al.*, 1988; Ushiyama *et al.*, 1989; Edwards *et al.*, 1990; Rasmussen *et al.*, 1995). The high cinnamic acid concentration induced a gradually withering of initial roots, which were superseded by neoroots at the end of the experiment. The phenol profile of initial roots of stressed ribworts was modified by the accumulation of glucosylated cinnamic acid derivatives, which were also detected in neoroots (Fons, 1998).

In this paper, *(E)*-cinnamic acid was added into the culture medium of younger *P. lanceolata* seedlings (30-day-old) in order to investigate if the plant age could modify their cinnamic acid detoxification ability. Content time course of the two major CGEs were carried out by HPLC from root ribworts. The temporary appearance of two new cinnamic derivatives (NCD) and structure elucidation of one of them are also reported.



Verbascoside : R=Rhamnose
Plantamoside : R=Glucose

Fig. 1.- Structures of plantamoside and verbascoside.
Fig. 1.- Structures du plantamoside et du verbascoside.

II. MATERIAL AND METHODS

Seeds of wild *P. lanceolata* were cultured in Murashige and Skoog's (MS) medium with 10 $g.l^{-1}$ agar under alternating 12 hour light/dark. Thirty-day-old ribworts were transferred, on day 0 of the experiment, in MS medium containing 1mM *(E)*-cinnamic acid dissolved in 0.1% DMSO and the experiment ran until day 30.

Every two-three days, from day 0 to day 30, residual cinnamic acid level in agar medium and roots, V and P contents from control and stressed plants (cinnamic acid fed plants) were evaluated by HPLC. Fifty seedlings were used for each experiment. CGEs and NCD extraction, and HPLC analyses were previously reported by Fons *et al.* (1998). Each experiment was carried out in triplicate and bars represented standard errors.

^{1}H and ^{13}C-NMR spectra of compounds **1** and **2** were taken in CD3OD at 400 MHz (Bruker AM400WB).

Ionisation spectra of compounds **1** and **2** were carried out with HP 5989 A (interface: HP Particle Beam LC/MS 59180 B; ionisation potentiel: 70 eV; source temperature: 250°C; Quaed temperature: 120°C).

III. RESULTS AND DISCUSSION

The biotransformation of *(E)*-cinnamic acid by thirty-day-old *P. lanceolata* was investigated for the first time on whole seedlings.

Results showed that most of cinnamic acid added to the culture medium of *P. lanceolata* disappeared quickly (Fig. 2). Only 35% of initial cinnamic acid amount were found in the agar at day 6 and less than 20% at day 15; 2% of this acid were still detected in the culture medium at day 21. No free cinnamic acid was detected in both initial and neoroots of stressed plants. However, the absorption of cinnamic acid by the seedlings was confirmed by some toxicity fingerprints on *P. lanceolata* roots: initial roots of stressed plants slowly degenerated as reported for 75-day-old seedlings (Fons *et al.*, 1998) and were gradually superseded by neoroots, appearing on day 8. These neoroots were investigated for CGEs by HPLC from day 15 to day 30 when the biomass was sufficient. V contents from control and stressed plants (both initial roots and neoroots) averaged at 8.5 ± 3 mg.g^{-1} dry weight. No difference was found for V content from the three root types (controls, initial roots and neoroots) all along the experiment (Fig. 3). P contents were more dispersed during the thirty days of the experiment (from 9 to 60 mg.g^{-1} dry weight); P level of initial roots from stressed ribworts was higher than P level of control roots (Fig. 3). High standard error values of P contents showed the results variability obtained for the initial roots of stressed plants. Then, cinnamic acid did not induce an increase of V root levels. No conclusion could be drawn for P accumulation. Further investigations with labelled precursors should be carried out to answer this question.

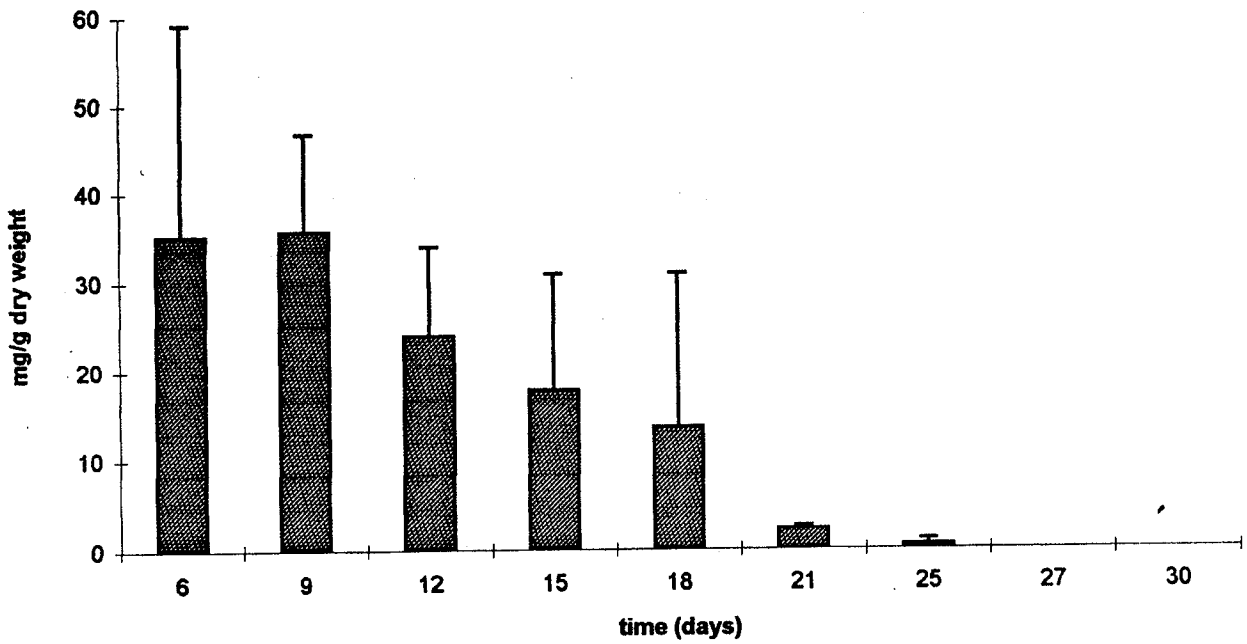


Fig. 2.- Time course of residual *(E)*-cinnamic acid content in culture medium of whole *P. lanceolata*.
Fig. 2.- Pourcentage d'acide *(E)*-cinnamique résiduel dosé dans le milieu de culture de plantes entières de *P. lanceolata*.

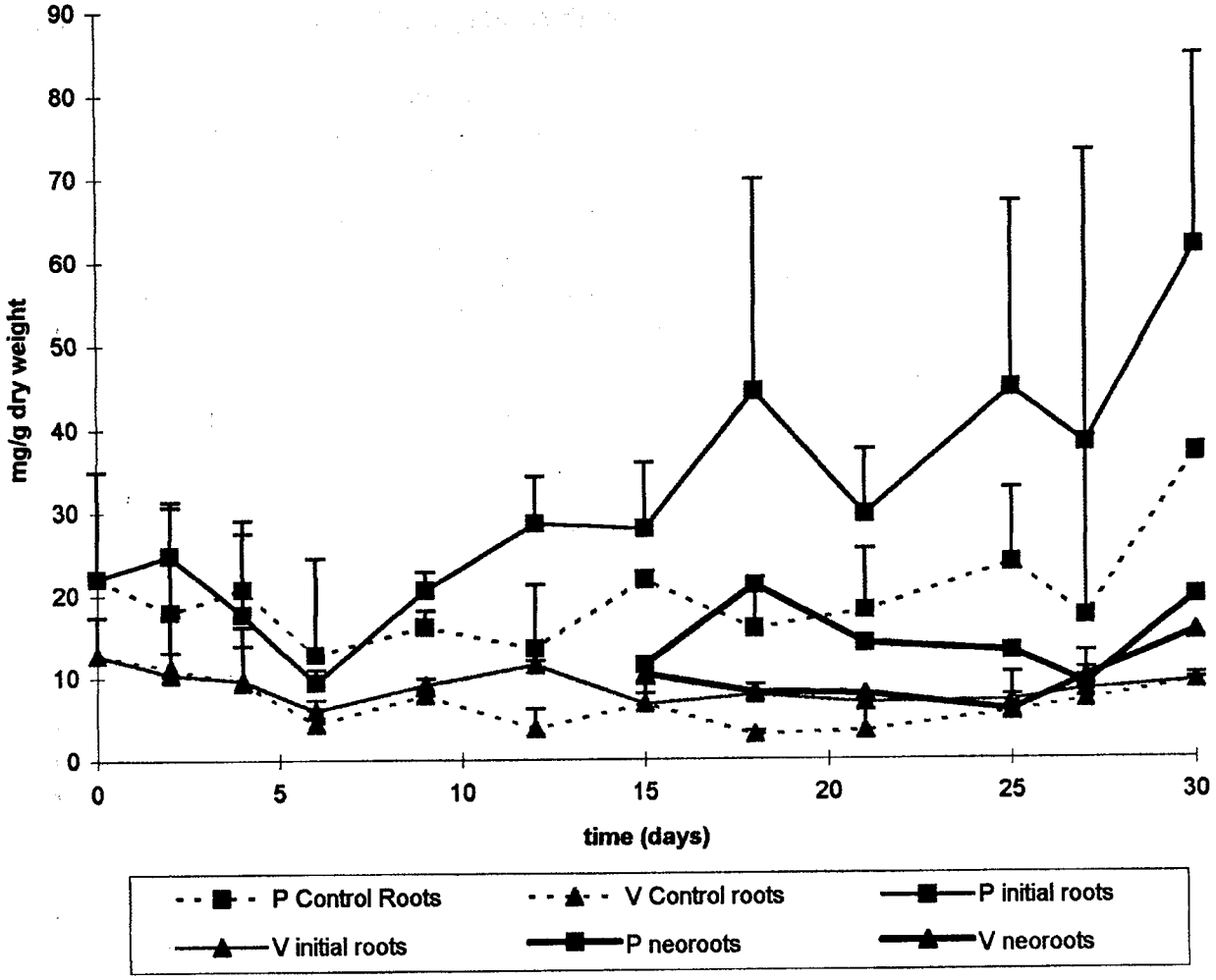


Fig. 3.- Time course of verbascoside (V) and plantamoside (P) contents in control roots and in both initial roots and neoroots of stressed plants during experiment on thirty-day-old whole seedlings.
Fig. 3.- Taux de verbascoside (V) et de plantamoside (P) dans les racines des plantes témoins, dans les racines initiales et les néoracines des plantes stressées au cours des expériences réalisées sur plantes de 30 jours.

On the other hand, phenolic profile of stressed roots was disturbed by the temporary appearance of two new cinnamic acid derivatives (NCD) detected by TLC and HPLC analyses. Structures of both NCD were partially identified by TLC and HPLC as *p*-coumaroyl-glucose (PCG) and feruloyl-glucose (FG) after acid and alkaline hydrolyses. Traces of the two *(Z)*-isomers of each acid were detected by HPLC after their irradiation with UV light (Fons *et al.*, 1998).

This paper reported the complete structure of *p*-coumaroyl-glucose which was isolated from seedling roots in sufficient quantity to allow RMN analyses. The ionisation spectrum of PCG similar to that of *(E)-p*-coumaric acid showed a peak at *m/z* 164 (data not shown), which confirmed the presence of this acid moiety. The structure of *(E)*-PCG was completely elucidated by spectral NMR analyses as *(E)-p*-coumaroyl-1-O-β-D-glucopyranoside (Fig. 4) [**1**].

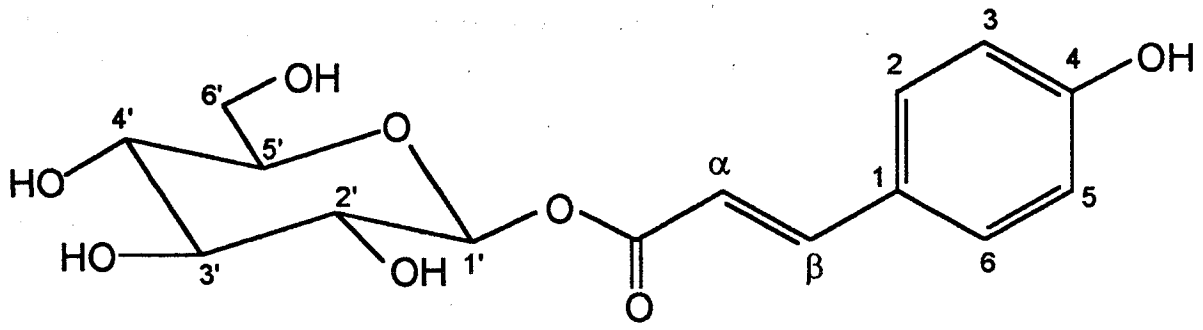


Fig. 4.- Structure of *(E)*-*p*-coumaroyl-1-O-*β*-D-glucopyranoside [**1**].
Fig. 4.- Structure du *(E)*-*p*-coumaroyl-1-O-*β*-D-glucopyranoside [**1**].

[**1**]- ^{1}H-NMR (CD_3OD) *δ*: 3.3-3.5 (4 H, H-2', H-3', H-4', H-5'), 3.68 (1H, *dd*, *J*=11.9 Hz ; 4.5 Hz, H-6'), 5.56 (1H, *d*, *J*=7.8 Hz, H-1'), 6.35 (1H, *d*, *J*=15.9 Hz, H-*α*), 6.81 (2H, H-3, H-5), 7.48 (2H, H-2, H-6), 7.71 (1H, *d*, *J*=15.9 Hz, H-*β*). ^{13}C-NMR (CD_3OD) *d*: 53.1 (C-6'), 60.1 (C-4'), 63.0 (C-2'), 67.0 (C-3'), 67.8 (C-5'), 84.8 (C-1'), 103.3 (C-*α*), 105.4 (2C, C-3, C-5), 115.9 (C-1), 120.4 (2C, C-2, C-6), 137.0 (C-*β*), 153.2 (C-4), 156.7 (C=O).

The structure of the mixed minor *(Z)*-PCG was also determined [**2**].
[**2**]- ^{1}H-NMR (CD_3OD) *δ*: 5.53 (1H, *d*, *J*=8.15 Hz, H-1'), 5.8 (1H, *d*, *J*=12.8 Hz, H-*α*), 6.74 (2H, H-3, H-5), 6.93 (1H, *d*, *J*=12.8 Hz, H-*β*), 7.72 (2H, H-2, H-6).

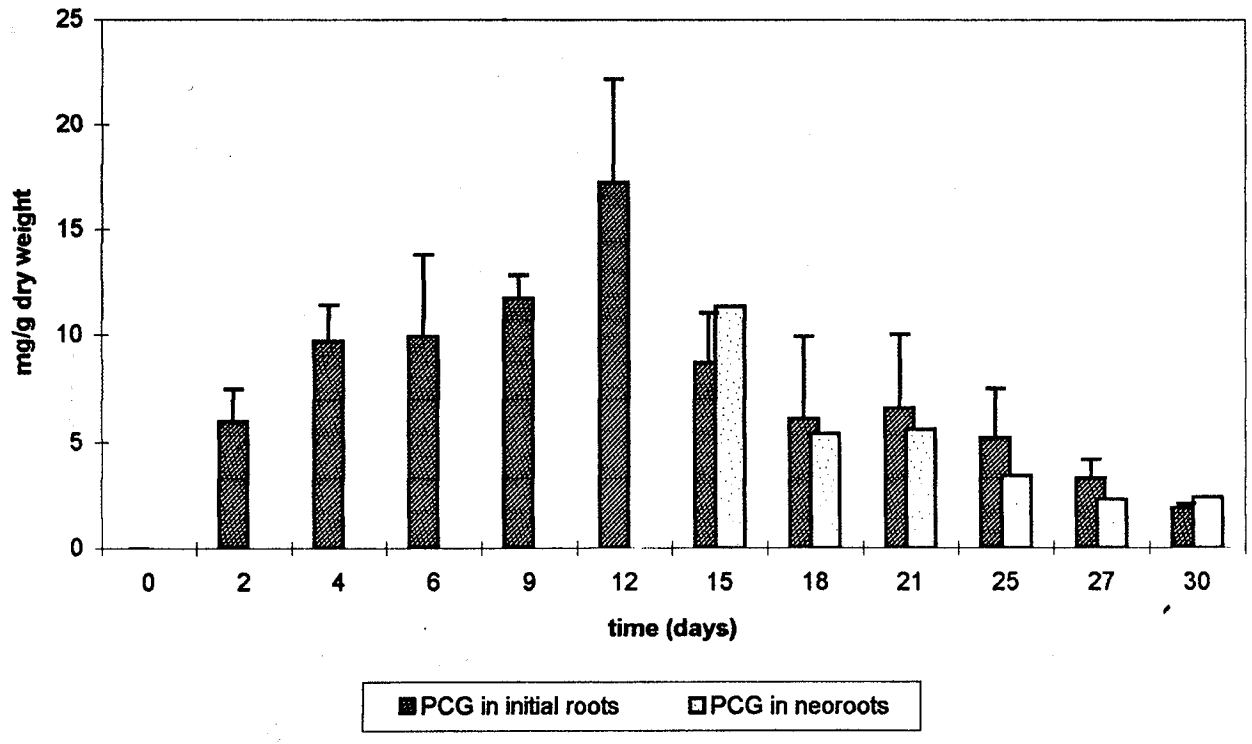


Fig. 5.- Time course of PCG contents in both initial roots and neoroots of ribworts fed with *(E)*-cinnamic acid.
Fig. 5.- Dosage des taux de PCG dans les racines initiales et dans les néoracines des plantules cultivées avec acide *(E)*-cinnamique.

PCG was early detected by HPLC from initial roots of stressed ribworts (Fig. 5) at day 2 (6 $mg.g^{-1}$ dry weight). PCG concentration increased during the first week of the experiment to reach the maximal levels at day 12 (17.2 $mg.g^{-1}$ dry weight) and then decreased to day 30 (1.9 $mg.g^{-1}$ dry weight). PCG was also detected in neoroots (Fig. 5) at high concentrations at day 15 (11.3 $mg.g^{-1}$ dry weight) and then gradually decreased until day 30 (2.4 $mg.g^{-1}$ dry weight). It is very interesting to note that PCG contents from stressed roots of 30-day-old ribworts were twice higher than those of 75-day-old ribwort whose maxima PCG accumulation in both initial and neoroots were only 7 $mg.g^{-1}$ dry weight at days 11 and 16, respectively (Fons *et al.*, 1998). We stated that the age of the seedlings influenced ribwort ability to accumulate PCG bioproduced after cinnamic biotransformation.

Exogenous aromatic precursor like benzoic acid, L-phenylalanine, *(E)*-cinnamic acid or *p*-coumaric acid added to various species plant culture media are generally biotransformed by glycosyl conjugation. Many authors reported two types of pathways: the first one, induced ether formation from alcohol and sugar moieties (Tabata *et al.*, 1988; Rasmussen *et al.*, 1996) and the second one induced the esterification of the sugar moiety from the carboxylic acid function (Molderez *et al.*, 1978; Ellis, 1983; Moriguchi *et al.*, 1988; Ushiyama *et al.*, 1989; Edwards *et al.*, 1990). The glycosyl conjugation increases the water solubility of outer precursors and allows their fast detoxification (Harborne, 1979; Luckner, 1990). Many plant cell cultures have been investigated for precursor biotransformation ability. The main drawback of cell suspension is their limited biosynthetic potential when compared with that of whole plants (Suga and Hirata, 1990; Stepan-Sarkissian, 1991). That is why we have chosen to investigate biotransformation of *(E)*-cinnamic acid by *P. lanceolata* whole seedlings.

IV. CONCLUSION

(E)-cinnamic acid was biotransformed by thirty-day-old whole *P. lanceolata* cultures to *p*-coumaroyl-glucose and feruloyl-glucose while plantamoside and verbascoside contents were not modified. The two new cinnamic derivatives were essentially stored in plant roots for fifteen days and then disappeared from chemical profile of roots. Maximum level of *p*-coumaroyl-glucose from thirty-day-old seedlings was higher than the content produced by seventy-five-day-old ribworts as previously reported (Fons *et al.*, 1998).

Based on the attractive results obtained from whole *P. lanceolata*, further investigations are in progress from transformed root cultures of ribworts derived upon infection with *Agrobacterium rhizogenes*. Indeed, the tumoral root cultures with high growth rate usually show high level productivity of secondary metabolites.

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