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# Germination of *Plasmodiophora brassicae* resting spores stimulated by a non-host plant

Hanna Friberg, Jan Lagerlöf and Birgitta Rämert
*Department of Ecology and Crop Production Science, Swedish University of Agricultural Sciences, P.O. Box 7043, SE–750 07, Uppsala, Sweden (Phone: +46-18-672651; Fax: +46-18-672890; E-mail: hanna.friberg@evp.slu.se)*

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**Abstract**

Plant-induced germination of *Plasmodiophora brassicae* resting spores was studied in a laboratory experiment. Spore reaction was analysed in nutrient solution with exudates from growing roots of different plant species – one host plant (*Brassica rapa* var. *pekinensis*) and four non-host plants (*Lolium perenne*, *Allium porrum*, *Secale cereale* and *Trifolium pratense*) – and in controls with distilled water and nutrient solution. It was found that root exudates from *L. perenne* stimulated spore germination more than exudates from the other plants, including those from the host plant. The effect could not be explained by differences in the nutritional composition of the solutions due to differential uptake of the plant species, or by differences in root activity, measured as exudation of soluble sugars. This is the first time such a separation of factors has been done in analysing the influence of plants on *P. brassicae* germination. Although stimulation of *P. brassicae* resting spore germination is not restricted to the presence of host plants, it seems to vary depending on the plant species.

## Introduction

One of the most destructive diseases of crucifers (Brassicaceae) is clubroot disease, caused by the protist *Plasmodiophora brassicae* (Braselton, 1995). Infection by the pathogen results in large gall formations on the roots. Diseased plants suffer from reduced uptake of water and nutrients, growth is affected and eventually the plants may die. *P. brassicae* survives in the soil as resting spores, which germinate into zoospores that can infect the root hairs of the host. The infection is extended into the root cortex, where large galls are produced. Inside the galls, new resting spores of the *P. brassicae* are produced. When the galls disintegrate at the end of the *P. brassicae* life cycle, the spores are liberated into the soil (Ingram and Tommerup, 1972). In the absence of a host plant, these spores are able to persist in the soil for extended periods of time. Wallenhammar (1996) found that the half-life of spores in Swedish soils averaged 3.6 years when no host was present, and calculated that the time needed for a highly infested soil to reach a spore concentration below the detection level was 17.3 years.

Germination of resting spores is presumed to be stimulated in the presence of a host plant, but germination also occurs in the absence of a host. This spontaneous germination varies with different environmental conditions. It is enhanced by increased humidity and temperature, reduced by alkaline pH, and also varies with concentration of other inorganic ions in the soil (Macfarlane, 1970; Takahashi, 1994a). Germinated spores with no

access to a host plant are considered to survive only short periods of time (Suzuki et al., 1992; Takahashi, 1994b). The possibility of stimulating germination and thereby accelerate spore reduction, in the absence of host plants is of great interest for controlling the disease. It has been suggested that, besides host plants, certain non-host plants have the ability to enhance germination. Zoospores have also been shown to be able to infect the root hairs of some plants, e.g. *Holcus lanatus*, *Lolium perenne* and *Tropaeolum majus* (Webb, 1949; Macfarlane, 1952). Cortical infection and the subsequent production of resting spores in plants other than crucifers have so far been found only in limited amounts in *T. majus* and *Beta vulgaris* (Ludwig-Müller et al., 1999).

The mechanisms of induction of spore germination has been attributed to several factors. Macfarlane (1970) proposes that a germination stimulant is excreted from plant roots. Suzuki et al. (1992) support this idea and characterise the germination-stimulating factor (GSF) as a heat stable, fairly polar and low molecular weight compound. The presence of this factor was found not only in host plants, but also in the non-host plant lettuce (*Lactuca sativa*). The authors suggest that the GSF is neither related to susceptibility nor restricted to crucifers. Kowalski and Bochow (1996), using other non-hosts plants, draw the same conclusion. In contrast, Narita and Nishiyama (1955) found that juice from crushed crucifer roots had a germination-stimulating effect on the spores, whereas juice from other plants was inhibitory.

The objective of this study was to examine the effect of different plant species on *P. brassicae* resting spore germination under laboratory conditions. Spore germination in reaction to exudates from a highly susceptible host plant (*Brassica rapa* var. *pekinensis*) was compared to germination in reaction to exudates from non-host plants. On the basis of the literature, three of the selected non-host plants (*Lolium perenne*, *Allium porrum*, *Secale cereale*) were considered to have a stimulating effect on germination of *P. brassicae* spores (Ikegami, 1985; Robak, 1994, 1996; Wallenhammar, 1999), while one species (*Trifolium pratense*) was considered not to have this effect (Robak, 1996; Wallenhammar, 1999). Spore germination was studied in nutrient solutions containing exudates from plant roots during growth. The covariance of plant nutrient conditions on spore germination, caused by differential uptake of nutrients, was included in the study.

## Materials and methods

### *Spore preparations*

*Plasmodiophora brassicae* was isolated from a heavily infested arable soil at Säbyholm (59.5° N, 17.6° E) 30 km NW of Stockholm in Sweden. Chinese cabbage (*B. rapa* var. *pekinensis* cv. Granaat, Bröderna Nelsons Frö) was grown in the soil for 6 weeks in the greenhouse according to the soil mix method of Castlebury and Glawe (1993). The diseased roots derived were washed and rotted at room temperature in plastic bags for 1 week. Spores were extracted and purified by gradient centrifugation as described by Suzuki et al. (1992).

### *Plant growth and root exudate solutions*

Five different plant species were used in the experiment: The host plant Chinese cabbage (*B. rapa* var. *pekinensis* cv. Granaat), and the non-hosts red clover (*T. pratense* cv. Sara, Swalöf Weibull), leek (*A. porrum* cv. Regius, Swalöf Weibull), perennial rye grass (*L. perenne* cv. Helmer, Swalöf Weibull) and winter rye (*S. cereale* cv. Amilo, Swalöf Weibull).

The plants were grown in liquid cultures with a circulating system where the solutions were adjusted 1–2 times per day, to compensate for the nutrient uptake of the plants. This was monitored by measurements and adjustments of conductivity (320 $\mu S\ cm^{-1}$) and pH (4.8) through addition of two stock solutions with different forms of nitrogen ($NH_4^+$ and $NO_3^{2-}$) and weight proportions: N 100, K 65, P 13, Ca 7, Mg 8.5, S 9, Fe 0.7, Mn 0.4, B 0.2, Zn 0.06, Cu 0.03, Mo 0.007, Na 0.003, Cl 0.03. The method of Ingestad and Lund (1986) was chosen to ensure well-defined growing conditions and to avoid excessive amounts of nutrients. The different plant species were grown in separate cultures in order to keep the root exudates separate. Since plant growth rate differed between the plant species studied, plants were grown until the root systems of the five plants grown together in one cup filled the apparatus growth cups (400 ml). The time to reach this volume was determined in

an earlier study (*A. porrum* 9 weeks, *T. pratense* 5 weeks, *L. perenne*, *S. cereale* and *B. rapa* var. *pekinensis* 4 weeks). Before the start of the last week, all cups were filled with fresh nutrient solution and each cup was adjusted separately four times per day. The solution from one cup was used as one experimental unit. Each treatment had 10 replicates.

*Observations on spore behaviour*

A 50 ml portion of nutrient solution with root exudates was poured into a 200 ml Erlenmeyer flask and purified spores were added to a concentration of $3\times10^6$ spores $ml^{-1}$. The flasks were covered with plastic film, shaken gently twice a day, and incubated at room temperature in darkness. A droplet (0.2 µl) of exudate solution containing spores was placed on a microscope slide and stained with orcein, whereafter the proportion of germinated spores was recorded under a light microscope (1000×), by counting empty spores as described by Naiki et al. (1987). The status of 200 spores (germinated or ungerminated) from two different microscope slides was analysed for each replicate. For each slide, 100 spores along a transect across the slide were analysed. If one transect was not enough to find 100 spores, two parallel transects were analysed. Spore germination in the exudate solutions was analysed every second day. At day 6, spore aggregation started in the solutions, making it harder to count individual spores, and after day 8 bacterial growth was observed. To avoid problems with determination of spore status and side effects caused by bacterial growth, only data from days 0, 2 and 4 were used in the statistical analyses.

*Characterisation of exudate solutions*

From each treatment, five randomly chosen replicates were chemically characterised. The concentrations of nitrogen (N; $NH_4^+$ and $NO_3^{2-}$), phosphorous (P), sulphur (S), calcium (Ca), magnesium (Mg), manganese (Mn), sodium (Na), and boron (B) were analysed using the Inductively Coupled Plasma Atomic Emission Spectroscopy (ICP-AES) technique. The concentrations of soluble sugars (glucose, fructose, mannose, and myoinositol) were analysed using ion chromatographic analysis. All chemical analyses were performed by the Department of Production Ecology, SLU.

*Data analysis*

Statistical analyses of spore germination were made using the ANOVA procedure and Tukey's test ($P<0.05$) for multicomparisons (MINITAB). Differences in chemical composition as compared to germination were analysed by a centred and standardised PCA (principle component analysis; SIMCA-P version 10.0, Umetrics AB; Anonymous 2002). The importance of treatments and the chemical variables measured was analysed with PLS (partial least square regression; SIMCA software). The proportion of spores germinated at day 4 was used as the response variable. In the PCA and PLS analyses, only the replicates that were analysed for chemical composition were used ($n=5$).

## Results

At the beginning of the spore reaction experiment (day 0), the percentage of germinated spores was close to 0 in all treatments. After 2 days, the spores in exudate from *L. perenne* showed a trend for a higher proportion of germinated spores compared with the other treatments. After 4 days, the response caused by *L. perenne* differed significantly from that of all other treatments. The host plant *B. rapa* var. *pekinensis* gave a response different only from the distilled water control, i.e. not significantly different from other non-host plants or the nutrient solution control. There were no significant differences among the other treatments (Figure 1).

There were differences among the treatments in the chemical variables measured (Table 1). Winter rye (*S. cereale*) had a higher concentration of Ca, Mg and Mn (Table 1). Most of the chemical parameters were only weakly correlated with resting spore germination. Boron concentration was slightly positively correlated and N-tot slightly negatively correlated with germination (Figure 2a, b).

The PLS analysis showed that the stimulatory effect of *L. perenne* treatment was the most important factor determining spore germination (Figure 3). The analysis resulted in two significant components, using in total 55% of the data, and with these was able to explain 70% of the variation

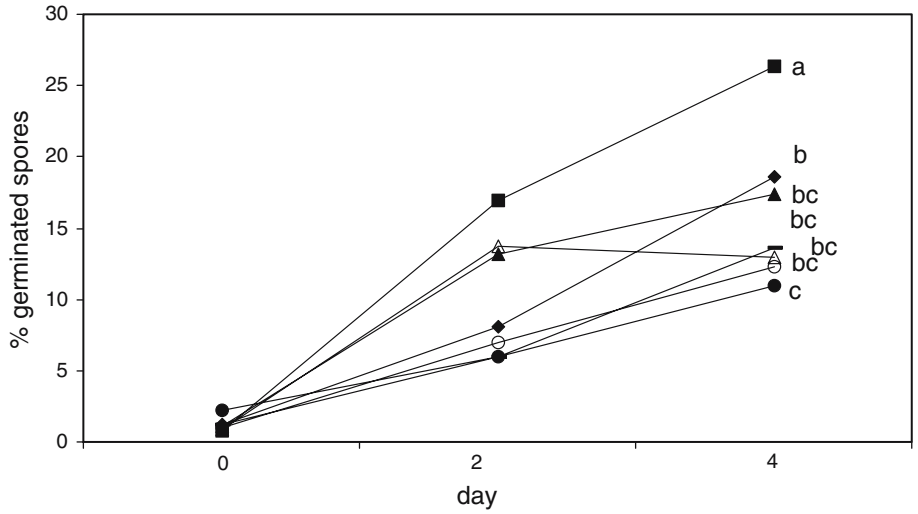


*Figure 1.* Percentage germinated spores of *P. brassicae* in exudate solution from perennial ryegrass (*L. perenne* ■), Chinese cabbage (*B. rapa* var. *pekinensis* ♦), leek (*A. porrum* ▲), red clover (*T. pratense* Δ), winter rye (*S. cereale* ○) and in controls: nutrient solution (–) and distilled water (●), at days 0, 2 and 4). Treatments denoted with the same letter (day 4) are not significantly different from each other ($n = 10$, $P < 0.05$).

in spore germination at day 4. The predictive ability of the model ($Q^2$) was 52%. Total N, Na and Mn concentration and *T. pratense* treatment were also highly significant variables, but with a negative effect on germination. The variables of moderate significance were *S. cereale* treatment (negative effect), boron (positive effect) and *B. rapa* var. *pekinensis* treatment (positive effect) (Figure 3).

## Discussion

In the present study, plant effects on *P. brassicae* resting spore germination were separated from other factors such as nutrient and sugar content with help of PLS analysis. If nutrient variation had been more important than plant treatment, this would have resulted in a higher ranking in Figure 3 of the important substance than of all plant treatments. In this study, however, *L. perenne* achieved the highest ranking, showing that this treatment had the greatest influence on germination. Sugar content, a measurement reflecting root activity, had no significant influence on the PLS model. In fact, it was one of the variables with the lowest VIP (Variable Importance on Projection) values. This indicates that root activity was not of vital importance for the outcome of the experiment.

From the PLS analysis, *T. pratense* was found to have a significantly negative influence on

*Table 1.* Chemical composition of root exudate solutions from plant treatments and controls used in the spore reaction experiment

| Treatment | N-tot (ppm) | P (mg $l^{-1}$) | S (mg $l^{-1}$) | Ca (mg $l^{-1}$) | Mg (mg $l^{-1}$) | Mn (mg $l^{-1}$) | Na (mg $l^{-1}$) | B (mg $l^{-1}$) | Sugar (mg $l^{-1}$) |
|---|---|---|---|---|---|---|---|---|---|
| *B. rapa* var. *pekinensis* | 7 (2.7) | 18 (3.9) | 11 (2.5) | 6 (0.8) | 10 (1.8) | 0.3 (0.04) | 1.1 (0.3) | 0.3 (0.06) | 8 (4.2) |
| *L. perenne* | 1 (1.1) | 18 (6.4) | 10 (4.3) | 8 (3.0) | 10 (4.3) | 0.3 (0.8) | 0.9 (0.6) | 0.5 (0.2) | 3 (1.0) |
| *T. pratense* | 6 (8.6) | 21 (10.7) | 14 (7.2) | 7 (3.3) | 11 (5.0) | 0.5 (0.2) | 6.5 (3.8) | 0.4 (0.2) | 8 (7.9) |
| *A. porrum* | 2 (0.5) | 18 (2.0) | 12 (2.5) | 6 (1.1) | 8 (1.7) | 0.3 (0.8) | 5.2 (1.2) | 0.4 (0.09) | 4 (1.2) |
| *S. cereale* | 12 (8.3) | 25 (3.0) | 15 (2.1) | 12 (1.1) | 17 (1.9) | 1.4 (0.3) | 7.1 (1.2) | 0.5 (0.07) | 8 (2.8) |
| Nutr. solution | 15 (0) | 16 (0) | 10 (0) | 8 (0) | 9 (0) | 0.4 (0) | 0.4 (0) | 0.2 (0) | 0 (0) |
| Dist. water | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |

Mean values (SD) $n = 5$.

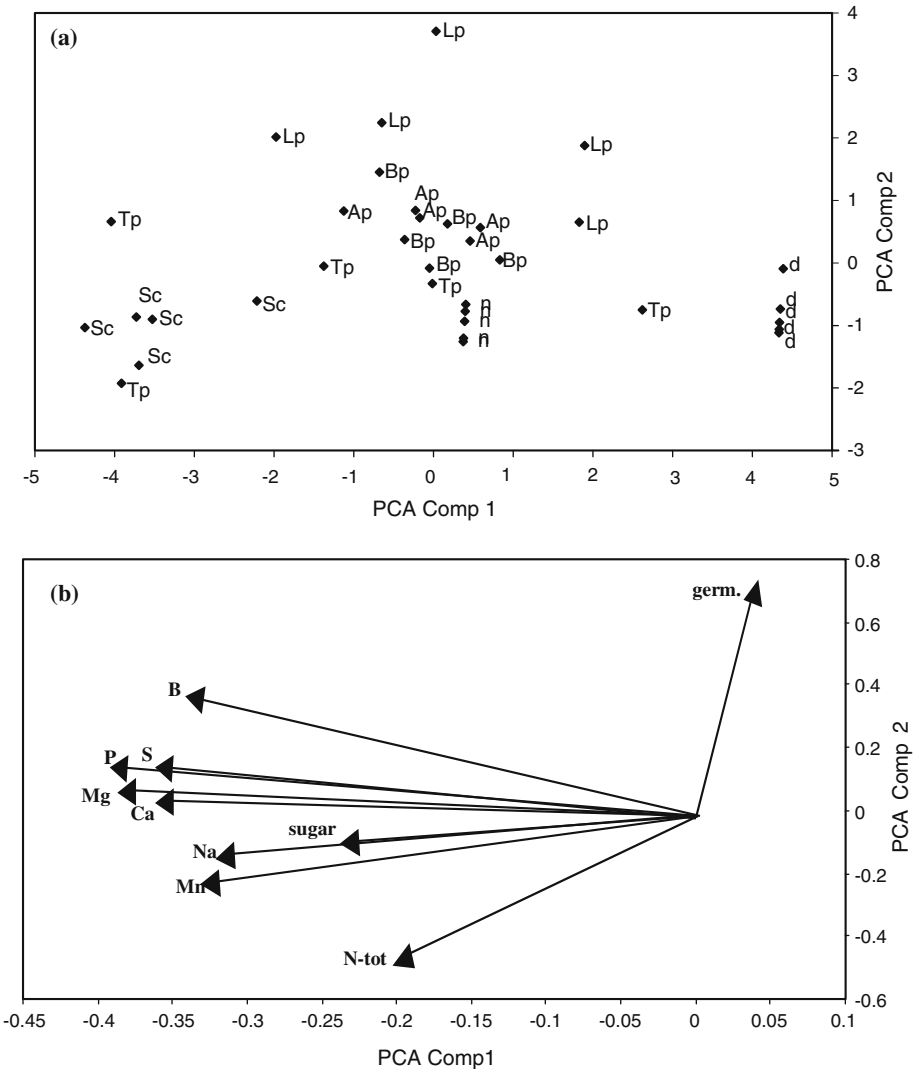


*Figure 2.* Principal Component Analysis of chemical composition for exudate solutions. (a) variation between samples of the different treatments: *B. rapa* var. *pekinensis* (Bp), *L. perenne* (Lp), *A. porrum* (Ap), *S. cereale* (Sc), *T. pratense* (Tp), nutrient solution (n) and distilled water (d). (b) variables explaining the variation in 2a: germination day 4 (germ.), chemical variables including soluble sugars (sugar) and total N concentration (N-tot). $n = 5$.

germination when effects of the chemical factors studied were accounted for (Figure 3). This is in accordance with results from field studies by Robak (1996) and Wallenhammar (1999). In contrast to the hypothesis, the non-host plant *L. perenne* stimulated germination of *P. brassicae* resting spores more than the host plant *B. rapa* var. *pekinensis*, which stimulated germination only compared to the distilled water control (Figure 1). Even after compensation for nutritional differences in the PLS analysis, *B. rapa* var. *pekinensis* had only a weakly significant influence on spore germination (Figure 3). Although many authors mention that spores of *P. brassicae* react specifically to the presence of host plant roots (Macfarlane, 1970; Takahashi, 1991; Wallenhammar, 1999), this was not observed in the present study, similarly to Suzuki et al. (1992) and Kowalski and Bochow (1996). Suzuki et al. (1992) found a GSF not only in exudate solutions from a susceptible cultivar of *Brassica campestris* but also from a resistant cultivar and a non-host plant (*Lactuca sativa*). Kowalski and Bochow (1996) observed stimulation from several hosts (*B. oleracea* of

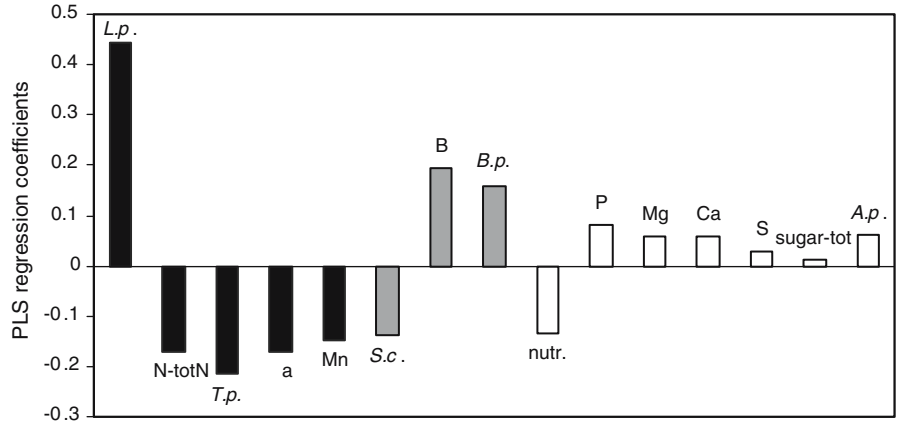


*Figure 3*. PLS regression coefficients showing direction and strength of variable influence on the model with germination at day 4 as the response variable. Bar colour shows the VIP (Variable Influence on Projection), with black bars as highly significant variables (VIP > 1.0), grey bars as moderately significant variables (VIP = 0.8–1.0) and white bars as variables with low influence (VIP < 0.8). Plant treatments in italics, *L. perenne* (*L.p.*), *T. pratense* (*T.p.*), *S. cereale* (*S.c.*), *B. rapa* var. *pekinensis* (*B.p.*), *A. porrum* (*A.p.*). Nutrient solution control (nutr.). Chemical variables as in Figure 2. $n = 5$.

different cultivars, *B. napus* and *B. rapa* var. *pekinensis*) and the non-host plants *Lycopersicon esculentum*, *Cucumis sativus*, *Allium cepa*, and *A. porrum*). On the other hand, Narita and Nishiyama (1955) found that sap from crushed roots of host plants (varieties of *B. rapa*) stimulated germination whereas sap from *L. esculentum*, *Triticum aestivum* and *Avena sativa* had an inhibitory effect. The hypothesis of host-specific germination stimulation of *P. brassicae* resting spores should be tested further before any general conclusions can be drawn.

N-tot ($NO_3^{2-}$ and $NH_4^+$), Na and Mn had a negative influence on germination, and B a slightly positive effect (Figure 3). In studies on soils suppressive to *P. brassicae*, Na concentration has been correlated to disease suppression (Höper and Alabouvette, 1996). B concentration has been correlated to reduced clubroot incidence. Dixon and Webster (1988) however, suggest that this reduction can be attributed to pathogen inhibition during root hair and cortical stages of the life cycle. *Plasmodiophora brassicae* may be initially stimulated to germinate by B, although the subsequent inhibition during infection could result in reduced club formation.

Spore reaction was measured only during the first 4 days. Based on Macfarlane's (1970) observations on spore germination under similar conditions, only small changes occur after day 4. Therefore, there is reason to believe that the results would have been the same if spore germination had been observed for a longer period.

From this study, no conclusions can be drawn about the exact mechanisms behind the germination stimulation by *L. perenne*. One, or several, germination-stimulating substances might have been released from the roots of the tested plants, as suggested by Suzuki et al. (1992), or other factors counteracted the effect in some of the treatments. The GSF described by Suzuki et al. (1992) was a fairly polar low molecular weight substance. The effect of other substances known to occur in root exudates, such as alkaloids, flavonoids and terpenes (Marschner et al., 2004), have not been tested for their effect on germination of *P. brassicae* spores. In addition, activity of microorganisms, e.g. their metabolites, might have been important for the outcome of the present experiment since the plants were not grown in a sterile environment.

The potential of *L. perenne* to stimulate germination of *P. brassicae* resting spores will now be tested further in the soil environment and under field conditions. Studying the behaviour of an organism in an unnatural environment – in this case a soil organism in aqueous solution in the laboratory – always involves the risk of changes in that behaviour due to an abnormal chemical or biological situation, making the results

inapplicable to a field situation. In this study, the problem of chemical differences was avoided by growing the plants with a controlled nutrient supply, and with the PLS analysis separating out the influence of plant treatments and some possibly important parts of the chemical composition of the solutions. This is the first time such a separation has been done in the analysis of plant influence on *P. brassicae* germination.

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