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A new approach to modelling the dynamics of oospore germination in *Plasmopara viticola*

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Abstract Oospores, the only overwintering structures of *Plasmopara viticola*, the causal agent of grapevine downy mildew, are the unique source of inoculum for primary infections in vineyards. We show that their germination dynamics depend on both climatic and endogenous factors. In particular, overwintering in controlled conditions suggests that low temperatures prolong the oospore germinability, while constant or gradually alternating water availability increases germination rates. However, wide fluctuations in germination percentage, observed both in naturally overwintered oospores, and under controlled conditions, indicate an important role for endogenous factors in the germination frequency. *Ad hoc* experimental assays and microscopic observations highlight an important role for calcium in the germination process. However, for a profound understanding of the biological mechanisms underlying oospore germination, mathematical models of the germination dynamics are needed. But, classical differential models of germination dynamics are, with current knowledge, prohibitive due both to the complexity of the underlying processes and knowledge incompleteness. Then, we propose a hybrid method derived from the integration of qualitative differential models and fuzzy systems.

Keywords Downy mildew · Calcium signalling · Grapevine · Mathematical modelling · System identification

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Introduction

The sexual reproduction of all members of the class *Peronosporomycetes*, currently classified within the *Straminipila*, results in oospore formation. The oospores are typical quiescent structures, assuring the survival of the fungal species in unsuitable environmental conditions. In particular, oospores often represent the resting inoculum of the downy mildew agents during winter, when no susceptible tissues of the host plant are available for infection. During the spring, oospore germination provides the viable inoculum for primary infections of both annual and perennial hosts. Depending on the climatic

conditions, downy mildews can cause serious damage, sometimes involving almost entire yield loss. Since 1878, European vineyards have experienced the destructive effects of *Plasmopara viticola* (Berk. *et* Curt.) Berl. and De Toni, the causal agent of grapevine downy mildew, inadvertently introduced from Northern America. The pathogen can infect both the leaves and the clusters of the European grapevine, *Vitis vinifera* L., causing early defoliation and necrosis of flowers and berries. Since severe epidemics of grapevine downy mildew are associated with frequent rainfall and moderate temperatures occurring throughout the grapevine vegetative season, *P. viticola* is more threatening in temperate regions, while high temperatures and reduced water availability during late spring and summer usually prevent the spread of the disease. During the winter, oospores are the only surviving structures of *P. viticola* and the occurrence of primary infections depends on their germination. Studies based on DNA microsatellite analyses showed that new *P. viticola* genotypes continuously colonize host tissues from May until August, implying that oospore germination occurs from bud burst until the beginning of veraison (Gobbin et al. 2005). Another indirect assessment of oospore germination, performed by determining their capacity to infect grape leaf discs in controlled optimal conditions (Hill 1998), revealed that oospores produce infectious macrosporangia until late June (Rossi et al. 2008b). Numerous investigations, carried out by directly assessing oospore germination percentages on water agar at 20°C from November until July, suggested that macrosporangium formation persists from mid–end November until the end of May, less frequently until the middle of June and sporadically until the end of June (Toffolatti 2007). Regular rainfall distribution usually has a positive effect on oospore germination rates, but abundant rain events were seldom associated with strong decrease in oospore germination percentages (Vercesi et al. 2000).

Although the contribution of the inoculum provided by surviving *P. viticola* structures to the final disease incidence is still unclear, oospore germination plays a key role in downy mildew epidemics. To the best of our knowledge, most of the efforts to model germination dynamics have assumed that only exogenous factors affect oospore germination. Different modelling approaches, ranging from regression (Rossi et al. 2002), mechanistic (Rossi et al. 2008a), neural networks (Vercesi et al. 2000), to fuzzy-neural models (Guglielmann et al. 2002), have recently been explored, and resulted in models that reconstruct the dynamics from temporal series of environmental and climatic variables, and are exploited to simulate either downy mildew primary infections or germination rates. Such models have limited predictive performance, and do not give any insight into the complex processes responsible for germination, since they do not consider the biological mechanisms underlying oospore germination and metabolic processes needed for macrosporangium formation. Thus, there is a need for mathematical models that describe the oospore germination dynamics in response to both endogenous processes and exogenous factors. The lack and/or incompleteness of knowledge of endogenous processes makes the formulation of structural dynamic models a difficult task.

The complex biochemical mechanism leading to oospore germination in *P. viticola* has been poorly investigated. On the other hand, it is well known that among the endogenous factors involved in spore germination, calcium plays an important role in zoosporogenesis (Judelson and Blanco 2005), zoospore motility (Donaldson and Deacon 1993a) as well as encystment and cyst germination in numerous genera closely related to *P. viticola* (Hardham 2007; Warburton and Deacon 1998). The Ca^{2+} signalling network is initiated by environmental cues, acting on membrane receptors and determining conformational changes in a GTP-binding protein. The G-protein activates phospholipase C, which in turn causes the hydrolysis of inositol-4,5-bisphosphate (PIP2) into two secondary messangers, diacylglycerol and inositol-1,4,5-trisphosphate (IP3). IP3, in turn, stimulates the release of Ca^{2+} through channels located in vacuoles and the endoplasmic reticulum. Higher cytoplasmic levels of Ca^{2+} lead to the activation of calmodulin, causing the activation of enzymes involved in several downstream processes (Berridge et al. 2003).

The aim of the present work is twofold: (1) to investigate the influence of water availability on oospore germination rates and the role of calcium in macrosporangium formation, and (2) to formulate a structural model of the dynamics of the oospore germination process in response to both exogenous and endogenous factors. In this paper a particular emphasis will be given to the former problem.

Materials and methods

Oospore germination dynamics

Overwintering oospores

Grapevine leaves showing downy mildew mosaic symptoms were collected in mid October in an untreated and naturally infected vineyard of cv Corvina located near Verona in the first year and of cv Pinot gris near Pavia in the second year. Symptomatic leaf zones were observed by a Leitz Orthoplan microscope at 40×, and areas showing high oospore concentration were carefully excised with a razor blade. To allow a rapid recovery of the oospores from the overwintering substrate, nine to twelve series of 70 nylon (100 μm pore size) bags, filled with 20 oospore rich leaf fragments, were prepared. Three sample series were overwintered in three different sites on the soil surface of the vineyard where the leaf samples were collected. The remaining sample series were overwintered in controlled conditions at 5°C on sand layers contained in seed trays that were kept: constantly water saturated (3 series), constantly dry (3 series) and, in the second year only, gradually alternating between dryness and saturation (3 series). A sample series for each overwintering condition was kept in a different thermostat. Adherence of the nylon bags, laying side by side on the vineyard soil and the sand, to the overwintering substrate was assured by using anti-hail nets. The meteorological data were collected in the overwintering vineyard by an automatic station (Siap+Micros, Olympo, Italy) with an hourly step.

Germination assays

Every three days from the beginning of November until the end of July, three samples per each overwintering condition were collected, and the oospores were isolated by finely grinding the leaf fragments in a small glass potter, containing a few ml of sterile distilled water. To eliminate larger leaf debris, the resulting suspension was filtered through a nylon net (100 μm pore size). After further filtration through a 45 μm pore size nylon net, the oospores were collected from the filter surface. A concentration of 5 oospores μl^{-1} was obtained by repeatedly rinsing and resuspending the oospores in sterile distilled water. Oospores were incubated in the dark at 20°C for 14 days in Petri dishes (4.5 cm in diameter) on water agar (Agar Noble, Difco, 1% w/v). Macrosporangium formation was checked daily with a Leica Wild M10 stereo microscope at 40× magnification. Percentages of germinated oospores (G) per sample were estimated on four replicates consisting of 100 oospores. Each germination assay was carried out on 1,200 oospores per overwintering condition.

Role of calcium in the germination process

Germination and Ca^{2+} signalling system

The involvement of calcium ions in the oospore germination process was first investigated using pharmacological agents able to interfere with different steps of Ca^{2+} signalling. All chemicals were purchased from Calbiochem (La Jolla, USA), apart from $CaCl_2$ and $LaCl_3$ (Sigma-Aldrich, USA). The calcium chelator EGTA was dissolved in 1 N NaOH and the pH of the solution adjusted to 7.5. The calcium channel blockers, $LaCl_3$ and methoxyverapamil, as well as $CaCl_2$ were dissolved in sterile distilled water. The calcium ionophore (A23187), the calmodulin antagonists (calmidazolium chloride, W5 and W7), the protein kinase inhibitors (K252a and KN93) and the calcineurin inhibitor (cyclosporin) were dissolved in dimethyl sulfoxide (DMSO), whose final concentration in water-agar was 0.2%. Neomycin sulfate, a phospholipase C (PLC) inhibitor, and TMB-8, an intracellular calcium release inhibitor, were dissolved in distilled water. The effect of the above listed chemicals was tested on oospores isolated from samples overwintered at 5°C on constantly water saturated sand. The germination assays were carried out as previously described on water agar amended with different concentrations of the chemicals (Table 1) and repeated twice. The negative controls were represented by water agar and water agar supplemented with 0.2% DMSO and with 0.01% NaOH. The chemicals used in the present study have previously been employed in investigations of the role of calcium in spore germination, hyphal growth, appressorium formation and toxin production in different fungal species (Ahn and Suh 2007; Chung 2003; Donaldson and Deacon 1993b; Judelson and Roberts 2002; Lee and Bostock 2006; Shaw and Hoch 2000; Torralba and Heath 2001; Uhm et al. 2003; Warburton and Deacon 1998).

Statistical differences between the oospore germination rates observed at the different concentrations of the chemicals were established by one-way ANOVA and multiple comparisons of the means with the REGW-F test, performed on transformed values of G, $\left(arcsin\sqrt{G/100}\right)$. Statistical analysis was carried out with the SPSS Statistics 17.0 software.

Calcium imaging and measurement

Ca^{2+} localization during the germination process was visualized by Fluo 3/AM (Calbiochem, USA). The membrane-permeable version of Fluo 3 is a non-ratiometric dye, essentially nonfluorescent without Ca^{2+}, which absorbs at 506 nm and emits at 526 nm upon Ca^{2+} binding. Fluo 3/AM was dissolved in DMSO in a 100 mM stock solution. The same suspension of oospores used for the germination assays was divided in two series and incubated at 20°C in double distilled water (ddH_2O) for 7 days, in order to obtain a high number of germinating structures. The oospores were then filtered through a nylon filter (45 μm mesh) and divided in two series: the first was resuspended in a phosphate-citrate buffer solution (41.2 mM Na_2HPO_4, 29.4 mM citric acid, pH 4.2), to evaluate the presence of autofluorescence; the second in the same buffer solution with 150 μM Fluo 3/AM and 2% Pluronic F127 (Sigma Aldrich), a nonionic dispersing agent that solubilizes large dye molecules in physiological media, to facilitate dye penetration (Takahashi et al. 1999). Oospores were incubated for 4 h on concave glass slides at room temperature and observed under an inverted Leica TCS SP2 AOBS confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany). Fluo 3/AM fluorescence was detected by using a 496 nm Ar/ArKr excitation laser line. To better discriminate between different fluorescence intensities, false colour images of the oospores during the germination process were generated by Adobe Photoshop 7.0 software.

The measurement of free Ca^{2+} concentration in the cytoplasm at the beginning of the germination process was carried out by estimating the dissociation constant (Kd) of the dye in the oospores. Five series of 1,200 oospores were incubated in ddH_2O for 7 days, washed with a buffer solution containing 50 mM EGTA, pH 7 and incubated for 25 h in solutions with increasing concentrations of free Ca^{2+} (0, 0.2, 0.5, 1 and 5 μM) and 100 μM A23187, the calcium ionophore, with the aim of equilibrating external and internal Ca^{2+} concentrations. After centrifugation (5 min, 1,200 rpm), the supernatant was discarded and oospores were resuspended in 150 μM Fluo 3/AM and 2% Pluronic F127 solution and incubated for 3 h at room temperature. Oospores were observed using a confocal microscope. Fluorescence intensity of four oospores per sample was measured by Image-J software (Abramoff et al. 2004). Kd under the experimental conditions and free Ca^{2+}concentration in the oospores incubated for 7 days in ddH_20 and in the Fluo 3/AM solution for 24 h were estimated as described by Tsien and Pozzan (1989).

The eventual presence of autofluorescence and the interference of A23187, which is characterized by intrinsic fluorescence (Kolber and Haynes 1981), were evaluated on 1,200 oospores kept in ddH_2O or in 100 μM A23187 without the addition of the dye.

A hybrid input-output modelling approach

The dynamics of oospore germination results from complex interacting mechanisms, the knowledge of which is mostly incomplete. This makes the conventional differential modelling problem difficult to address. However, purely input-output approaches, applied to model oospore germination dynamics by exploiting data related to past average values of germination rate and climatic parameters, such as temperature, rain and humidity, have demonstrated limited generalization properties and lack of interpretative capabilities (Guglielmann et al. 2002).

With the aim of formulating a robust and biologically interpretable quantitative model of germination dynamics, that accounts for both endogeneous processes and exogenous factors, a *hybrid* method, called FS-QM (Bellazzi et al. 2001; Guglielmann and Ironi 2005), which lies between the structural and input-output approach has been exploited. FS-QM uses the available, although incomplete, a priori structural knowledge to produce a good estimate of the unknown nonlinear map $y = f(\underline{x}, \underline{\theta})$ between the input variables $(\underline{x})$ and the output one (y). The method is domain-independent, and derives from the integration of Qualitative Models, represented in the QSIM formalism (Kuipers 1994),

and Fuzzy Systems (Wang 1994), hence, the name FS-QM. The QSIM approach allows the qualitative formulation of structural models despite the incompleteness of knowledge, and the derivation of all the qualitative behavioural distinctions of the dynamics of the system under study from one of its initial states. However, QSIM is not efficient when dealing with systems in which the dynamics result from interacting mechanisms. As this is the case for oospore germination dynamics, where endogenous factors regulate the germination process, a revised version of QSIM, enriched by a "divide-and-conquer" strategy for properly and efficiently simulating dynamics of complex systems (Guglielmann and Ironi, submitted), has been exploited.

Many classes of fuzzy systems have been proved to be excellent candidates for identification purposes (Jang 1993; Takagi and Sugeno 1985) as (1) they hold the universal approximation property (Wang 1994), (2) they are able to handle data samples, and (3) they are able to exploit qualitative and uncertain a priori knowledge of unknown system dynamics, which is expressed by inferential linguistic information in the form of IF-THEN rules. The latter property makes it possible to combine the QSIM and fuzzy approximation frameworks: the dynamics of the system derived from the simulation of the qualitative structural model is exploited to automatically generate a fuzzy rule-base, where each rule may be seen as a measure of the possible transition from system states to the next ones. The mathematical interpretation of the rule-base defines both the complexity and the analytical expression of the nonlinear fuzzy approximator $y = f(\underline{x}, \underline{\theta})$, which is identified with the desired accuracy by the tuning of the vector of parameters $\underline{\theta}$ to the input-output data. The approach, sketched in Fig. 1, embeds the a priori knowledge of the system at study into the input-output model, and consequently model robustness and interpretability are improved.

We observe that FS-QM, although it can be classified as an input-output predictive tool, preserves the interpretation capability offered by structural models. For example, its potential in hypothesis testing could be efficiently exploited in the context of oospore germination both for exploring different assumptions on endogenous processes and for simulating the effects of different treatments.

Results

Germination dynamics of *P. viticola* oospores

First year

During the first overwintering season, only 275 mm of rain were recorded in the vineyard (Fig. 2a). Frequent and usually light rain events occurred from November until the middle of January, followed by a long dry period lasting until the beginning of April. Subsequent rainy days characterized the first half of April, while only a few isolated showers were recorded in the following period. The average daily temperature in the vineyard ranged from 5 to 15°C in November and, generally from 0 to 5°C from December until the end of February. Daily temperatures below 0°C were detected only for a few days during the winter. Starting from March the temperature showed an almost steady increase and from June onwards was constantly higher than 25°C.

Oospores overwintered in the vineyard formed the first macrosporangia at the beginning of November (Fig. 2b). The germination rate increased until the end of December, showing wide fluctuations thereafter. In particular, a strong decrease in germination percentage was observed in the assay carried out immediately after rainfall in the middle of January, followed by a sudden and notable increase, resulting in the highest germination rate attained by the oospores overwintered in vineyard (17.04%). In the following period, increases in germination percentages were observed in oospores collected either before or after rainfall. The oospores overwintered in natural conditions did not germinate from the middle of June onwards.

Oospores, overwintered at 5°C on a sand layer kept constantly dry, germinated intermittently from November until July (Fig. 2b). Furthermore, macrosporangium formation occurred at a very low rate, ranging from 0.06% to 0.59%.

Oospores, overwintered at 5°C on a sand layer kept constantly water saturated, germinated from November until July (Fig. 2b). Their germination percentages increased until the beginning of January. Significant fluctuations in the germination rates, analogous to those observed in naturally overwintered oospores, were detected in the following germination assays. In comparison with the samples overwintered in the vineyard, significantly higher values were found from

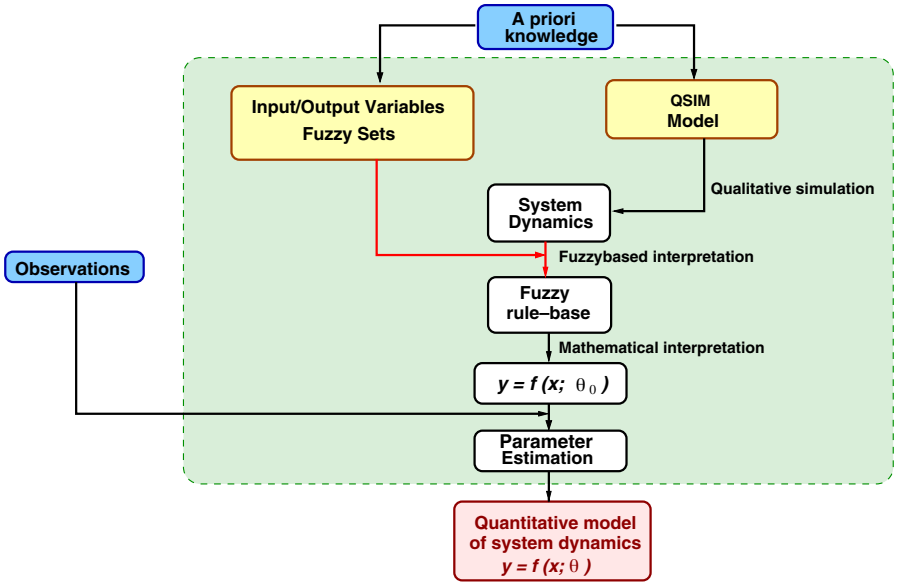

Fig. 1 Main steps of the FS-QM approach

the middle March until the end of July, except at the beginning of May. The highest germination rates for the constantly watered oospores were recorded at the beginning of March (24.4%) and May (27.53%).

Second year

From November until July, the total rainfall amount recorded in vineyard was 399 mm (Fig. 2c). The rain registered in November and December occurred predominantly at the beginning and at the end of both months. Only 10 mm of rain were recorded in January, while in the second half of February almost 50 mm fell in nine consecutive rainy days. After a few isolated rainy events, registered in March and in the first half of the following month, 42 mm of rain was recorded in the four last days of April. In May, June and July, sporadic showers, attaining sometimes 10–20 mm, interrupted a substantially dry period. From November to February the average daily temperature ranged generally from 0 to 5°C. Daily temperatures below 0°C were seldom recorded at the end of December, more frequently in the second half of January and for a few days at the beginning of February. From March onwards the daily temperature steadily increased, and exceeded 10°C in April. Average mild temperatures, only occasionally higher than 25°C, were recorded in vineyard in the following months, and sudden decreases were observed during or immediately after rainfall.

The germination rate of the oospores overwintered in vineyard increased from the end of November until the beginning of January (Fig. 2d). Afterwards the oospore percentage showed a fluctuating trend and attained its highest value (14.54%) at the middle of February. The abundant rain recorded at the end of February was associated with a sudden decrease followed by a significant increase of the oospore germination percentage, which remained almost stable until middle March. While at the end of March only 0.27% of the incubated oospores were able to form macrosporangia, in April the germination percentage ranged from 3.53 to 8.71. After a rapid increase observed at the beginning of May, the oospore germination rate constantly decreased and the last macrosporangia were observed during the germination assays carried out at the middle of May and in mid June.

The oospores kept on dry sand at 5°C attained the highest germination percentage (1.51%) at the beginning of December (Fig. 2d). Their germinability progressively decreased during the overwintering period, but even in July very few oospores were still able to germinate.

Until the second half of March, the germination trends of the oospores kept both on the vineyard soil and on a sand layer gradually alternating between dryness and saturation were similar, even if the oospores maintained in controlled conditions showed sometimes

higher fluctuations in germination rates (Fig. 2d). A very low germination percentage was assessed for the oospores kept in all the various overwintering conditions around the 20th of March. Starting from the end of April, the oospores kept in gradually alternating overwintering conditions showed germination percentages higher than those assessed for the oospores overwintered in vineyard. Furthermore, germination continued until the end of the experimental assays, although at a highly variable rate. The highest germination rates were attained at the end of March (20.74%) and during the last days of April (21.82%).

The oospores kept on a constantly water saturated sand layer showed an overall germinability and evident fluctuations generally higher than those assessed for the oospores exposed to natural and alternating overwintering conditions (Fig. 2d). The highest values of the germination rates for the constantly water supplied oospores were recorded on the 19th April (36.89%) and the 10th of May (32.76%). Lower germination percentages (circa 30%), were observed later on, the 19th June and at the beginning of July.

Role of calcium in the germination process

Germination and Ca^{2+} signalling system

The average germination rate of the oospores of *P. viticola* incubated on water agar at 20°C was 20.29% (Table 1). The final concentrations of DMSO and NaOH added to the medium had no discernible effect on oospore germination percentage (19.2% and 19.7% respectively).

The influence of exogenous Ca^{2+} was investigated by increasing its availability in the extracellular environment. Addition of $CaCl_2$, ranging from 100 μM to 10 mM, to the incubation medium had no significant effect on the oospore germination (Table 1). On the contrary, germinability significantly decreased at 100 mM $CaCl_2$. The Ca^{2+} chelator EGTA did not significantly reduce the germination

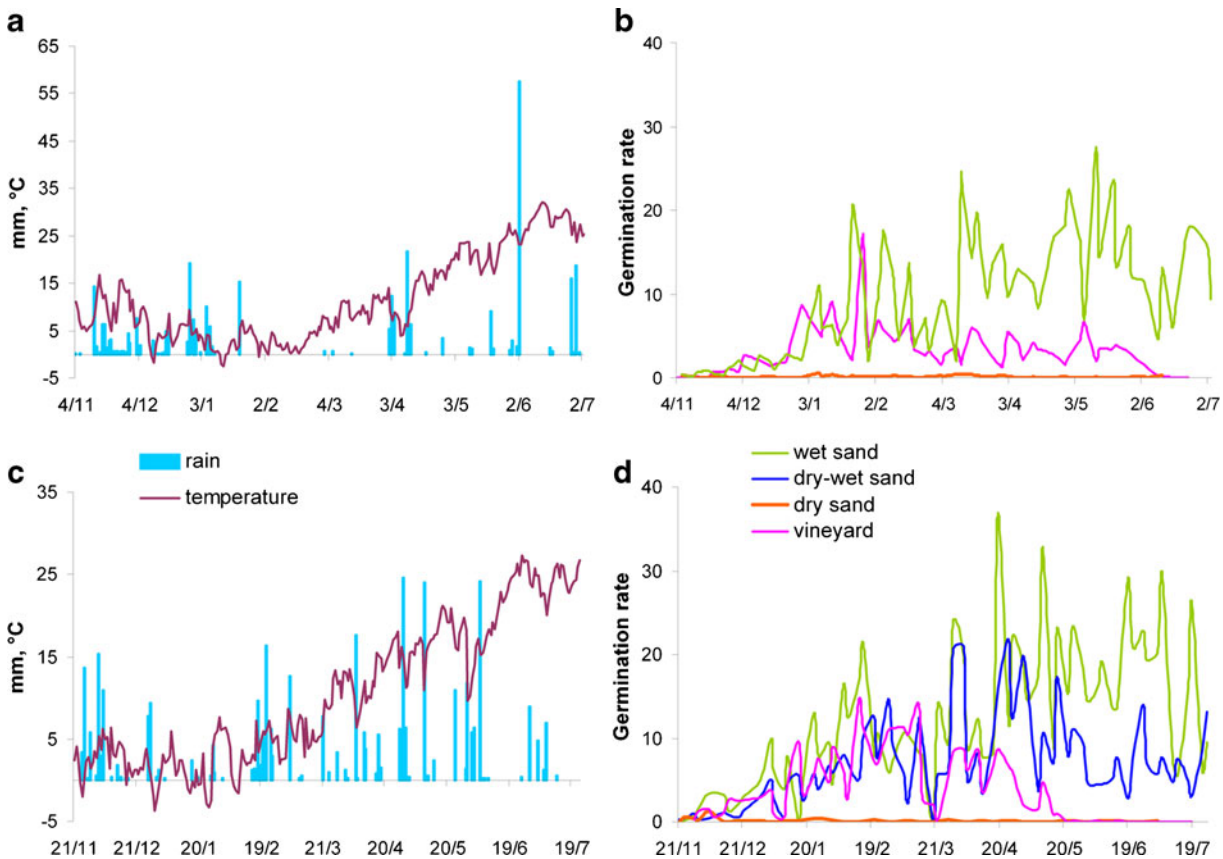

Fig. 2 Climatic conditions and germination dynamics of the oospores during the first (**a**, **b**) and second year (**c**, **d**) of investigation

rates at 100 μM, but at 1 and 10 mM strongly affected the ability of the oospores in differentiating macrosporangia. The Ca^{2+} ionophore A23187 significantly increased the oospore germination percentages, in particular when coupled with 100 μM $CaCl_2$.

Three Ca^{2+} channels modulators, TMB8, metoxyverapamil and $LaCl_3$ were tested (Table 1). La^{3+} displaces Ca^{2+} from superficial binding sites and blocks transmembrane fluxes on Ca^{2+}: when added to the water agar at 100 μM it completely inhibited oospore germination, while no significant effect was observed at the lower concentrations (Table 1). TMB8, an intracellular calcium release inhibitor, severely affected oospore germination at 100 μM and totally inhibited the process at 1 mM. The highest concentration, 1 mM, of methoxyverapamil, a blocker of the calcium channels located in the plasmamembrane, had a similar effect while oospore germination was less affected by the presence of 0.1 mM of the same channel blocker.

The involvement of phosphoinositide signalling system in oospore germination was investigated using neomycin sulfate, a phospholipase C inhibitor which completely suppressed macrosporangium formation both at 100 μM and 1 mM concentrations.

Increase in cellular Ca^{2+} level leads mainly to the activation of calmodulin (CaM), a Ca^{2+} binding protein, resulting in the formation of a Ca^{2+}-calmodulin complex (Ca-CaM) which in turn activates Ca-CaM dependent enzymes (Berridge et al. 2003). Calmodulin antagonists such as calmidazolium, W5 and W7 compete with Ca^{2+} for binding the protein, inhibiting calmodulin activation (Chung 2003). All the antagonists affected germination, but, while W5 and W7 strongly reduced the germination rates starting from 25 μM, calmidazolium showed the same effect only at a higher concentration (100 μM).

The Ca^{2+}/calmodulin-dependent protein kinase inhibitors, K252a and KN93, totally suppressed the oospore germination at respectively 10 and 100 μM. The same results were achieved using 100 μM and 1 mM of cyclosporin, which inhibits the Ca-CaM activated phophatase calcineurin.

Calcium imaging and measurement

Observations carried out at the confocal microscope showed that the calcium level in the cytoplasm of the quiescent oospores was particularly low and barely detectable (Fig. 3a). On the contrary in the early germination phase the cytoplasmic compartment contained a large amount of free Ca^{2+}, while in the central ooplast its concentration was much reduced (Fig. 3b). In comparison with the quiescent oosporse, the germinating sexual structures of *P. viticola* were characterized by thinner walls and a smaller ooplast, due to the mobilization of storage compounds. In more advanced germination stages (not shown) the central ooplast was no longer observable and oospore walls were progressively dissolving: Ca^{2+} was detected only in the cytosol at a relevant concentration (not shown). The emergence of the germ tube from the oospore was associated with a gradual translocation of Ca^{2+} through the growing hypha (Fig. 3c). After the beginning of sporangium formation, the oospore appeared completely empty. In developing sporangia, Ca^{2+} concentration varied greatly from zone to zone, and was particularly high in some membrane bound vesicles, probably due to cytoplasm cleavage leading to zoospore differentiation (Fig. 3d).

Autofluorescence was not detectable in oospores incubated in Ca^{2+} depleted water, while fluorescence intensity progressively increased in germinating oospores incubated at gradually higher Ca^{2+} concentrations (Table 2). The Ca^{2+} concentration in germinating oospores incubated in ddH_20 water was estimated using the Kd calculated from the calibration curve (R^2= 0.92) obtained by measuring the fluorescence intensity of Fluo-3/AM in the cytoplasm of the oospores incubated in water containing the following increasing concentrations of free Ca^{2+}, 0, 0.2, 0.5, 1 and 5 μM. In the described experimental conditions, 2.6 μM free Ca^{2+} was detected in the germinating oospores.

A FS-QM model of oospore germination dynamics

With the final goal of formulating a robust and interpretable model of the oospore germination process that accounts for the structural knowledge of endogenous biological mechanisms, the FS-QM method was applied to build an input-output relation $y = f(\underline{x}, \underline{\theta})$ between the input variables $\underline{x}$ and the output y, where $\underline{\theta}$ denotes the vector of parameters.

To reconstruct f according to the scheme given in Fig. 1, the available incomplete a priori structural knowledge of endogenous processes was firstly exploited. More precisely, it was assumed that calcium metabolism plays a crucial role in the activation of the

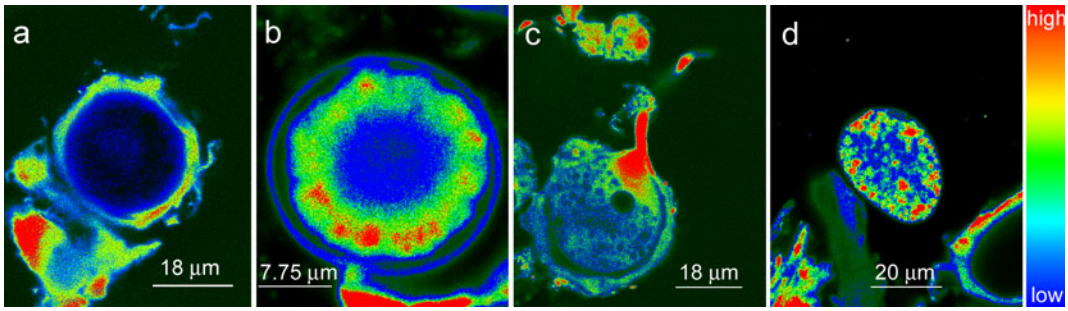

Fig. 3 Pseudocoloured confocal images representing Ca^{2+} levels (colormap[1]) during quiescence (**a**), at the beginning of germination (**b**), emergence of germ tube (**c**) and macrosporangium differentiation (**d**). [1] Low intensities of fluorescence/levels of Ca^{2+} are represented in blue, progressively higher in green, yellow and red

germination process, as schematically illustrated by the compartmental scheme given in Fig. 4.

The germination process, represented in the figure within the dashed rectangle, can be described by the following Ordinary Differential Equations (ODE):

$$\frac{dO_L}{dt} = -f_1\left(O_L, Ca_{cyt}\right) - f_2(O_L) \quad (1)$$

$$\frac{dO_G}{dt} = f_1\left(O_L, Ca_{cyt}\right) \quad (2)$$

The dynamics of latent oospores O_L is determined by two processes: the transformation into germinating oospores, modelled by $-f_1$, and the physiological loss of oospores, described by $f_2(O_L)$, monotonic increasing function of O_L.

The function $f_1(O_L, Ca_{cyt})$ models the germination rate governing the dynamics of germinating oospores O_G (Eq. 2). Under the assumption that germination starts when the concentration of cytosolic calcium, Ca_{cyt}, exceeds a given threshold Ca^*, the function f_1 is equal to zero when $Ca_{cyt} \leq Ca^*$, and it is monotonically increasing with both O_L and Ca_{cyt},

Table 1 Germination rates of the oospores incubated at different concentrations of the chemicals and results of statistical analysis (REGW-F)[a]

Chemical	Concentration									
	0	0,1 µM	1 µM	10 µM	25 µM	50 µM	100 µM	1 mM	10 mM	100 mM
$CaCl_2$	20.29 a						19.82 a	20.91 a	16.79 a	4.19 b
EGTA	20.29 a						20.76 a	0.21 b	0 b	
A23187	20.29 a						29.40 b			
A23187+Ca	20.29 a						32.09 b			
$LaCl_3$	20.29 a		13.71 a	14.22 a			0 b	0 b		
TMB 8	20.29 a			18.95 a			0.96 b	0 c		
Methoxyverapamil	20.29 a			18.88 a			10.34 b	0 c		
Neomycin sulfate	20.29 a			22.56 a			0 b	0 b		
Calmidazolium	20.29 a		19.56 a	24.26 a			7.53 b			
W7	20.29 a		22.35 a	16.87 a	5.66 b	0.65 c	0 c			
W5	20.29 a		22.35 a	15.91 ab	6.89 bc	0.79 cd	2.68 cd			
K252a	20.29 a	19.51 a	7.43 b	0 c						
KN93	20.29 a		19.28 a	1.53 b			0 b			
Cyclosporin	20.29 a		15.63 a	17.06 a			0 b	0 b		

[a] different letters correspond to significant statistical differences (α=0.05)

Table 2 Average fluorescence intensities (F) measured in the cytoplasm of germinating oospores incubated in different solutions. A: no addition of Ca^{2+}, ionophore (A23187) nor fluorochrome (Fluo 3/AM) to detect autofluorescence; B: addition of A23187 to evaluate the potential fluorescence of the ionophore; C-G: addition of both ionophore and fluorochrome at increasing concentrations of free Ca^{2+} to calculate the Kd of the dye; H: sample incubated in double distilled water in presence of the fluorochrome to calculate free Ca^{2+} concentration in active oospores before the germ tube differentiation

Sample	Free Ca^{2+} concentration (μM)	A23187 (100 μM)	Fluo 3/AM (150 μM)	F[a]
A	–	–	–	7.2 (a)
B	–	+	–	5.3 (a)
C	0	+	+	12.3 (a)
D	0.2	+	+	16.3 (ab)
E	0.5	+	+	22.8 (b)
F	1	+	+	24.4 (b)
G	5	+	+	38.4 (c)
H	–	–	+	31.2 (bc)

[a] A statistically significant difference corresponds to different letters (α=0.05)

when $Ca_{cyt} > Ca^*$. More precisely, $f_1(O_L, Ca_{cyt})$ can be expressed by the product of two functions $f_L(O_L)$ and $f_{cyt}(Ca_{cyt})$. The former one, $f_L(O_L)$, is a monotonic function of O_L. The latter function $f_{cyt}(Ca_{cyt})$, representing calcium release from the cell for the activation of the germination process, is equal to zero when $Ca_{cyt} \leq Ca^*$, and it is monotonically increasing with Ca_{cyt}, when $Ca_{cyt} > Ca^*$.

Calcium metabolism is characterized by a complex mechanism of uptake from (f_{12}) and release into (f_{21}) internal reserves of calcium in response to an external input Ca_{ext}, corresponding to calcium absorption from the environment, and by the calcium release for germination: Fig. 5 shows the compartmental scheme of such a complex process, that can be described by the following differential equations:

$$\frac{dCa_{cyt}}{dt} = Ca_{ext} + f_{12}(Ca_{res}, Ca_{cyt}) - f_{21}(Ca_{cyt}) - f_{cyt}(Ca_{cyt}) - f_{out}(Ca_{cyt}) \quad (3)$$

$$\frac{dCa_{res}}{dt} = f_{21}(Ca_{cyt}) - f_{12}(Ca_{res}, Ca_{cyt}) \quad (4)$$

where Eqs. (3) and (4) describe the dynamics of cytosolic calcium and calcium reserves (Ca_{res}), respectively; Ca_{ext} is assumed to be constant; the fluxes f_{12}, f_{21}, between the two compartments, and f_{out}, denoting the physiological calcium losses, are nonlinear functions of their arguments.

Although the experimental study allows us to define the mathematical structure of the ODE equations, their complete formulation is hampered by the incompleteness of the current available knowledge of functional relationships as well as of kinetic parameter values in both models of calcium metabolism and the germination process in *P. viticola* oospores. Nevertheless, the functional relationships between variables are known in terms of regions of monotonicity: this kind of qualitative information can be successfully exploited to formulate a qualitative model according to the QSIM formalism (Kuipers

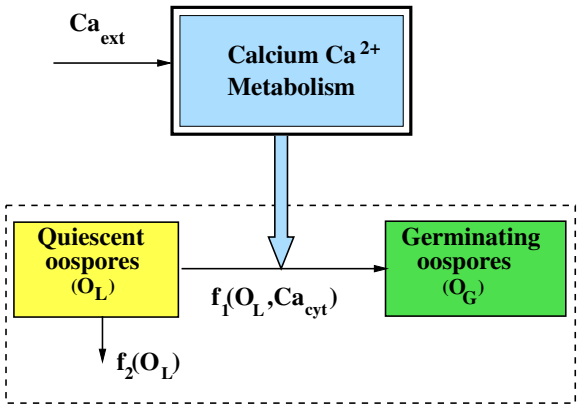

Fig. 4 The compartmental representation of oospore germination dynamics: the germination process in the dashed rectangle is regulated by calcium metabolism, whose control action is represented by the double arrow

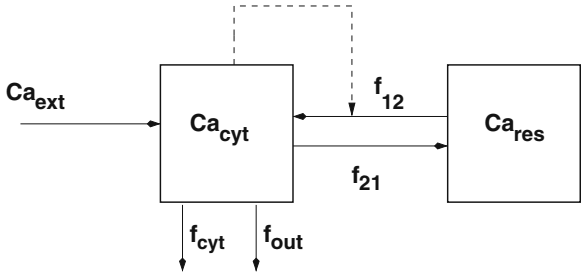

Fig. 5 Compartmental scheme of calcium metabolism

1994). The modelling formalism offered by QSIM has great potential to express incomplete knowledge of the functions and the levels, but its associated simulation algorithm, at least in its basic form, has an intrinsic limitation to scale-up to a complex system, such as the one under study, as it produces a very large and complex behavioural description that makes its translation into the fuzzy rules quite inefficient. With the aim of generating a tractable set of behaviours, the basic simulation algorithm has been enriched by a *divide-and-conquer* strategy (Guglielmann and Ironi, submitted). The qualitative simulation of the model provides in one run all the possible sound distinct qualitative behaviours of the system under study. The number of behaviours generated with the updated algorithm (one hundred) is computationally quite tractable, and allows the splitting of the generated behaviour tree into classes of equivalent behaviours, as for the input-output variables. A further significant improvement of the efficiency of the next step in the identification procedure is obtained by considering only one representative for each class: Fig. 6 exemplifies a possible class of behaviours that do not present qualitative significant distinctions as for input-output variables.

Then, the whole range of simulated system dynamics is encoded, through a mapping defined in (Bellazzi et al. 2001; Guglielmann and Ironi 2005), into the fuzzy approximator f under the form of fuzzy rules. A proper mathematical interpretation of the fuzzy rules allows us to build f, which is given by the following expression:

$$f(\underline{x},\underline{\theta}) = \frac{\sum_{j=1}^{M} \widehat{y}_j \prod_{i=1}^{n} \mu_i^j(x_i,\underline{\theta}_i^j)}{\sum_{j=1}^{M} \prod_{i=1}^{n} \mu_i^j(x_i,\underline{\theta}_i^j)} \quad (5)$$

where M is the number of the generated fuzzy rules, and n the number of input variables. The right-hand side of Eq. (5), whose derivation is out of the scope of this paper, was defined in Guglielmann and Ironi (2005) in accordance with the mathematical interpretation of the fuzzy operators in the rules given in (Wang 1994). The parameters $\underline{\theta}$ are then tuned to the data, and the germinating oospore dynamics is approximated by $\widetilde{O}_{G_{t+1}} = f\left(O_{G_t}, Ca_{cyt_t}; \underline{\theta}\right)$, where the components of the input vector $\underline{x} = \left(O_{G_t}, Ca_{cyt_t}\right)$ refer, respectively, to germinating oospores and cytosolic calcium concentration measured at time t, and $\widetilde{O}_{G_{t+1}}$ is the predicted value of germinating oospores at time $t+1$. For a complete quantitative formulation of the quantitative model of *P. viticola* oospores germination, the vector of parameters $\underline{\theta}$ needs to be estimated: at the current state, the tuning of parameters is hampered by the lack of temporal series of cytosolic calcium concentrations.

Discussion

Oospore germination depends primarily on the achievement of maturation, involving the fusion of gametangial nuclei, the formation of thick walls and

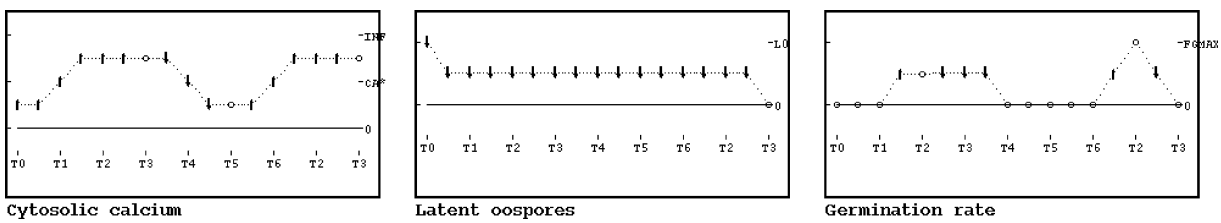

Fig. 6 Plot of a qualitative simulated behaviour of the relevant variables of the oospore germination model. More precisely, the time course of the variables Ca_{cyt}, O_L, and of the germination rate f_1 are plotted. *CA** denotes the calcium threshold above which germination is active. Calcium, and, consequently, germination rate exhibit an oscillating behaviour

nutritional reserves. Ultrastructural studies have shown that *P. viticola* oospores are mature by the middle of November (Vercesi et al. 1999). In fact the germination of the oospores overwintered in natural conditions occurs from the end of November onwards, continuously until mid May and then sporadically until mid June. Low temperatures and abundant rainfall occurring during the overwintering period have often been associated with high germination rates (Zachos 1959; Burruano and Ciofalo 1990; Serra and Borgo 1995), but the relationship between climatic conditions and germination dynamics needs to be more thoroughly investigated: in fact the germination percentage often showed notable decreases immediately after rainy events. Overwintering carried out in controlled conditions demonstrated that water availability affects oospore germinability: oospores kept at low temperature on dry substrate germinate at very low rates, but retain, to a certain extent, their viability as shown by the few germinated oospores detected throughout the overwintering season. On the contrary, oospores kept at 5°C at both alternating and constant water availability germinate more abundantly and for a longer period in comparison with those overwintered in the vineyard. The prolonged germination is probably due to the low temperature as previously demonstrated by Burruano et al. (1990). The germination rate of the oospores overwintered in natural and controlled conditions greatly diverges from March onwards: constant water supply is associated with the highest germination rates, attained in the last overwintering period. The positive effect of water availability on the overall oospore germinability is also demonstrated by the higher average germination percentage observed in the vineyard in the presence of higher rainfall (Fig. 2d). Increasing water availability also results in higher average and punctual germination rates but does not prevent the occurrence of wide fluctuations observed in every overwintering condition apart from dryness. In natural and controlled water supplied conditions, fluctuations in the germination rate occur almost synchronously suggesting that an increase in water availability can modulate germination, but does not interfere with its dynamics. Therefore exogenous factors such as temperature and rain do not completely account for observed variations in germination rates, but exert their influence together with endogenous factors which seem to be responsible for oscillations in germination capability.

Since a complex series of calcium controlled events are involved in the germination of the asexual spores of genera closely related to *P. viticola*, it seemed reasonable to hypothesize that calcium plays an important role in oospore germination. Experimental results indicate that oospore germination depends on both the uptake from extracellular compartments and the release from intracellular reserves of calcium. As further supply of extracellular calcium does not increase germination rates, oospores could re-absorb the previously released ion as demonstrated in the germinating cysts of *Pythium* and *Phytophthora* species (Deacon and Donaldson 1993). Following increases in cytosolic calcium, a Ca^{2+}-CaM complex is formed and in turn activates a large set of proteins, such as kinases and phosphatases, essential for macrosporangium formation as demonstrated by using specific protein inhibitors (Table 1). Despite the fact that pharmacological inhibitors of Ca^{2+} and CaM may lack target specificity, the use of a wide array of inhibitors provides an initial indication of the involvement of Ca^{2+} and CaM signalling system in oospore germination.

Furthermore calcium imaging was consistent with the involvement of calcium in the germination process. Moreover, the ion concentration in the cytoplasm of the oospores in the first germination stages is higher than the resting calcium level, typically 50–100 nM, usually found in fungal cytoplasm (Zelter et al. 2004). The results obtained by the experimental activity discussed above suggested that calcium does indeed play a key role in the germination process. Although still incomplete, the knowledge disclosed by the observations obtained during the experimental assays on quiescent and germinating oospores is sufficient to formulate the qualitative model described in this paper. The qualitative model has a great validity in itself as it can be exploited to test different hypotheses on both several aspects of calcium metabolism and the influence of exogenous factors on the overall process, and its simulation outcomes can suggest and drive further experimental activity aiming at gaining more and more refined information on variables involved. However, the quantitative predictions of oospore germination, that are our final goal, can be obtained only by the FS-QM model built from the qualitative

one, given temporal series of germinating oospores and cytosolic calcium concentrations. The quantification of Ca^{2+} in latent oospores requires further investigation as the measurements obtained so far are not sufficiently reliable to be used for the estimation of the parameters in the FS-QM model, and to fully identify germination dynamics. For this reason, alternative molecular approaches, such as the expression of the genes regulated by calcium, are necessary to more precisely describe the calcium signalling system during oospore germination.

Unlike purely input-output approaches, the identified hybrid model, which takes into account the endogenous mechanisms that regulate oospore germination, can be easily extended to deal with a more realistic context where water availability is considered. The model could be exploited as an experimental tool to simulate the germination dynamics in response to different levels of uptake/release of Ca^{2+} and/or different water availability regimes. More importantly, its simulation could highlight previously unobserved behaviours that could suggest new modelling hypotheses and/or new experimental designs essential to drive possible model revisions. Such revisions, necessary if simulated behaviours do not match the observed ones, will lead to a reliable model that completely explains the observed oscillating behaviours.

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