# Interaction between the mycoparasite *Verticillium fungicola* and the vegetative mycelial phase of *Agaricus bisporus*

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Host-parasite interactions of *Agaricus bisporus* and *Verticillium fungicola* were studied in paired-cultures. Different growth reactions were found according to the culture medium used, but no apparent alterations could be detected by light and electron microscopy in *A. bisporus* vegetative mycelia. *In vitro*, different hydrolytic enzymes are produced by *V. fungicola* in cultures with *A. bisporus* vegetative mycelial cell walls as the only carbon source. *In vitro*, this enzyme preparation is able to partially digest isolated *A. bisporus* vegetative mycelial cell walls. These results suggest that although apparent attachment of the mycoparasite to the host cell surface seems to occur, there is no *in vivo A. bisporus* vegetative mycelial cell wall degradation.

## INTRODUCTION

Dry bubble caused by *Verticillium fungicola* is still considered to be one of the most important diseases of the cultivated mushroom *Agaricus bisporus*. The mycoparasite produces several macroscopic symptoms in its host, including dry bubble, necrotic lesions and stipe blow-out.

Infected mushrooms have been studied using light and electron microscopy. The relation between *V. fungicola* and *A. bisporus* seems to be of an invasive necrotrophic nature. The parasite penetrates host cell walls by the combined effect of wall-lytic enzymes and mechanical pressure (Dragt *et al*. 1996, Calonje *et al*. 1997).

White *A. bisporus* fruit bodies have a low effective natural resistance to *V. fungicola* in contrast to some brown or wild strains (Dragt *et al*. 1995). Control of this disease has been partially achieved by the use of chemical products, cultural and sanitary practices. Apparently *V. fungicola* infects *A. bisporus* fruit bodies but does not affect the vegetative mycelium (Fletcher, White & Gaze 1986), so some authors speculate that the resistance of the vegetative mycelium to the infection process is lost when it aggregates in basidiomes. The purpose of this report is to study the interaction between *V. fungicola* and the vegetative mycelial phase of *A. bisporus*.

## MATERIALS AND METHODS

### *Organisms and culture conditions*

Commercial *A. bisporus* strain (smooth white Fungisem H25) was grown on Raper medium (Raper, Raper & Miller 1972). The cultures were incubated for two weeks at 25 °C statically, and the vegetative mycelium was harvested by filtration followed by washings with distilled water. *V. fungicola* var. *fungicola* (no. 161 from the collection of the CIES, Centro de Investigación, Experimentación y Servicios del Champiñón, Quintanar del Rey, Cuenca, Spain) isolated from naturally infected *A. bisporus* (H25) fruit bodies, was grown at 25° and maintained on Raper agar. For some interaction experiments compost extract agar was used (compost, 50 g $l^{-1}$, extracted with water at 120° and filtered through paper).

### *Enzyme assays, substrates employed and detection of 1,3-β-glucanase and chitinase activities on PAGE*

To prepare mycelial cell walls, *A. bisporus* vegetative hyphae were disrupted by the action of a Polytron homogeniser and the walls purified as described by Avellán, García Mendoza & Novaes-Ledieu (1986). The cell walls were freeze-dried and stored for further utilisation.

To produce enzyme, *V. fungicola* growth was carried out on the minimal synthetic medium (MSM) described by Calonje *et al*. (1997). The medium was supplemented with 0·1% (w/v) of *A. bisporus* vegetative mycelial cell walls and $NH_4NO_3$ as carbon and nitrogen sources. The remaining conditions for enzyme production have been described elsewhere (Calonje *et al*. 1997).

All enzyme assays, substrates employed as well as the detection of 1,3-β-glucanase and chitinase activities on PAGE have previously been described (Calonje *et al*. 1997).

To measure lytic activity of the enzyme extract on *A. bisporus* vegetative cell walls, mycelium was suspended in 50 mM sodium acetate buffer pH 5 (5 mg $ml^{-1}$) and incubated with the enzyme solution (3 mg $ml^{-1}$) in the same buffer. The mixture was incubated at 37° for 16, 24 and 72 h. The lytic

effect was evaluated microscopically and quantified by gravimetric analysis.

### *Hyphal interactions*

Hyphal interaction between *V. fungicola* and *A. bisporus* vegetative mycelia was studied in dual-cultures using a technique similar to that of Nordbring-Hertz (1982). Autoclaved discs of cellophane (9 cm diam) were placed aseptically on the surface of two different culture media, Raper agar and compost extract agar, in 9 cm Petri dishes. The fungi were inoculated at opposite ends of the cellophane membrane. Due to the different growth rate of the two fungi, *A. bisporus* was inoculated 7 d before the *V. fungicola* conidial suspension to insure that the interaction took place in the middle of the membrane. The mycoparasite and *A. bisporus* grew towards each other and their hyphae intermingled.

In order to eliminate the possible influence of the culture media in the interaction between the vegetative mycelia of *A. bisporus* and *V. fungicola*, the basidiomycete was also grown on compost extract agar covered with a cellophane membrane. After two weeks at 25°, the membrane covered with the *A. bisporus* mycelium was placed in an empty Petri dish and inoculated with a conidial suspension of *V. fungicola* prepared by gently scraping the surfaces of 1 wk old colonies in tap distilled water. Both mycelium and conidial suspension were incubated for 4 d at 25° in a wet chamber.

### *Light and electron microscopy*

For light microscopy, strips of cellophane (1·5 $cm^2$) cut from the zone of intermingled hyphae of both fungi were fixed in 2% (w/v) glutaraldehyde in 0·1 M phosphate buffer pH 7 for 1 h at room temperature. The samples were washed three times with phosphate buffer and examined using a Zeiss light microscope.

For TEM, samples of mycelia from the interaction region of dual-cultures of *A. bisporus* and *V. fungicola* (*A. bisporus* mycelium grown for 7 d before inoculation with a *V. fungicola* conidial suspension) in cellophane strips (5 $mm^2$) as well as samples of *A. bisporus* vegetative cell walls incubated at different time periods with *V. fungicola* enzyme solution were fixed in 2% (w/v) glutaraldehyde in 0·1 M phosphate buffer pH 7 for 4 h at room temperature. The samples were washed four times with phosphate buffer and postfixed in 2% (w/v) osmium tetraoxide in the same buffer for 1 h at room temperature. After several washes, the samples were dehydrated in ethanol and embedded in Vestopal W resin. Ultrathin sections (50 nm) were collected on 300-mesh copper grids coated with Formvar and stained with uranyl acetate and lead citrate. Samples were examined in a Philips EM300 transmission electron microscope.

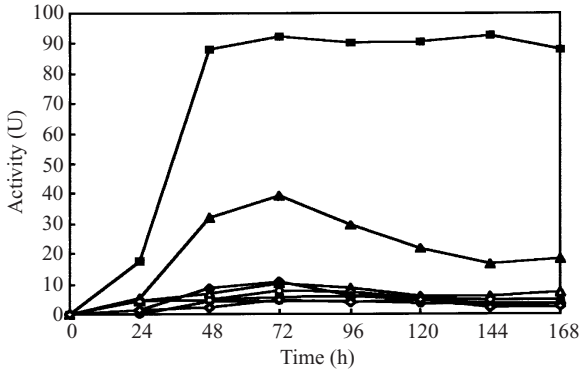



**Fig. 1.** Time-course curves of enzyme activities (U) produced by *V. fungicola* growing in MSM supplemented with 0·1% *A. bisporus* vegetative mycelium cell walls: (■) 1,3-β-glucanase; (□) 1,4-β-glucanase; (▲) 1,6-β-glucanase; (△) 1,3-α-glucanase; (●) 1,4-α-glucanase; (○) 1,4-β-glucanase; (◆) N-acetylglucosaminidase.

## RESULTS

### *Enzyme production of* V. fungicola *grown on MSM medium with* A. bisporus *vegetative mycelial walls*

Fig. 1 shows the time course curves of 1,3-β-glucanase, 1,4-β-glucanase, 1,6-β-glucanase, 1,3-α-glucanase, 1,4-α-glucanase, 1,4-β-xylanase and N-acetylglucosaminidase produced by *V. fungicola* grown in MSM supplemented in 0·1% *A bisporus* vegetative mycelium cell walls as the only carbon source. Although all these activities were detected in the culture filtrate, production of 1,3-β-glucanase and 1,6-β-glucanase was significantly higher compared to the other assayed activities. The highest enzyme activities were obtained after

**Table 1.** Extracellular enzyme activities produced by *V. fungicola* grown in MSM supplemented with 0·1% *A. bisporus* vegetative mycelial walls. Enzyme activities were measured after 72 h incubation. Results are means of at least three experiments. U, total activity (mmol $h^{-1}$); U $mg^{-1}$, specific activity.

| Enzyme activity | Substrate | U | U $mg^{-1}$ |
|---|---|---|---|
| 1,3-β-glucanase | Laminarin | 92·2 | 602·9 |
| 1,6-β-glucanase | Pustulan | 39·2 | 256·2 |
| 1,4-β-glucanase | CM-cellulose | 5·6 | 36·9 |
| 1,3-α-glucanase | *A. nidulans* 1,3-α-glucan | 10·1 | 66·1 |
| 1,4-α-glucanase | Amylose | 4·2 | 27·7 |
| 1,4-β-xylanase | Xylan | 7·7 | 50·7 |
| β-D-glucosidase | *pNP*-β-Glc | 14·6 | 95·7 |
| α-D-glucosidase | *pNP*-α-Glc | 0·1 | 0·6 |
| β-D-xylosidase | *pNP*-β-Xyl | 0·1 | 0·6 |
| β-D-mannosidase | *pNP*-β-Man | 0·1 | 0·6 |
| Chitinase | Chitin | 4·3 | 28·2 |
| N-Acetylglucosaminidase | *pNP-GlcNAc* | 10·6 | 69·3 |
| Chitobiosidase | *pNP*-$(GlcNAc)_2$ | 3·9 | 25·6 |
| Protease | Casein | 459·9 | 3003·0 |

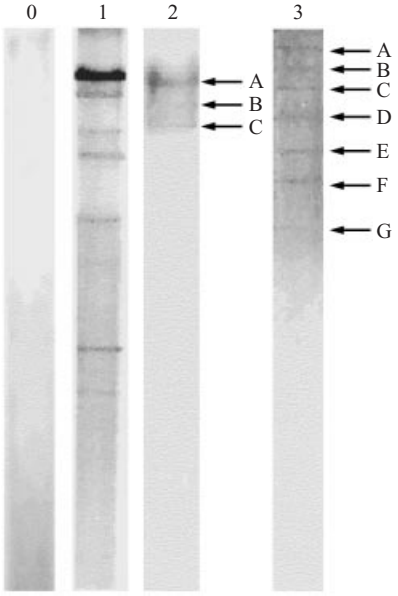
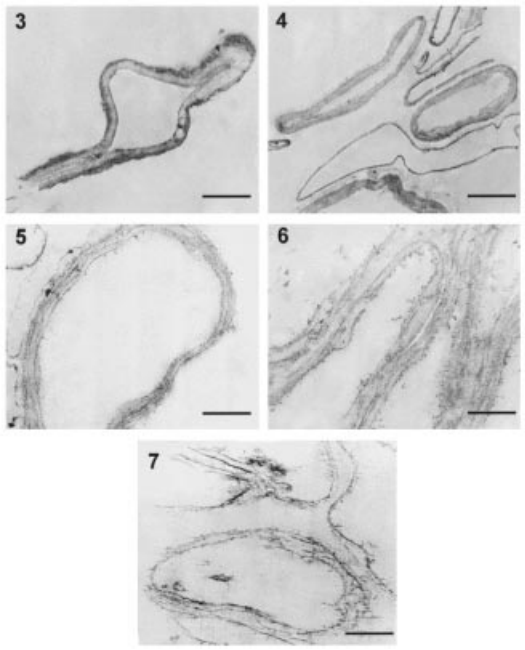
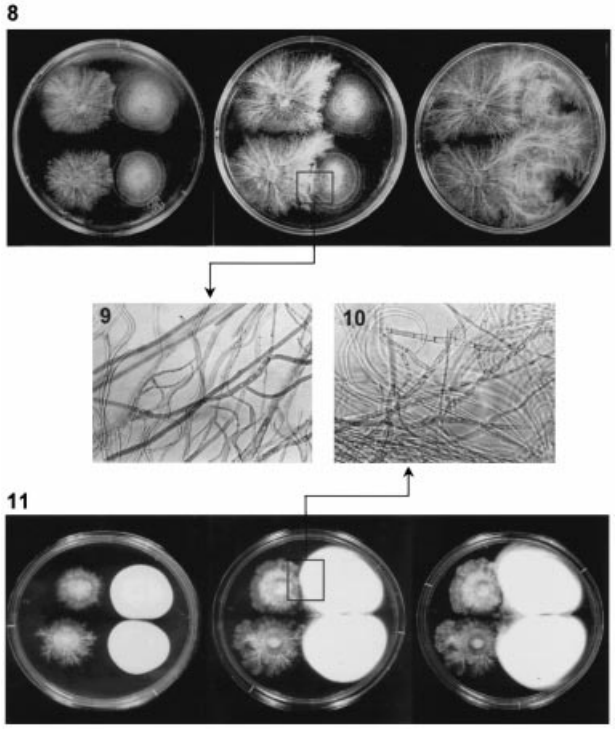



**Fig. 2.** PAGE (6–15% gradient): Lane 1, extracellular proteins (80 µg) coomassie staining; Lane 2, β-1,3-glucanase activities (20 µg) and lane 3, chitinase activities (20 µg) secreted by *V. fungicola* grown on MSM supplemented with *A. bisporus* vegetative mycelium cell walls. Non-inoculated medium containing *A. bisporus* cell walls was used as control (lane 0).




**Figs 3–7.** TEM micrographs of *A. bisporus* vegetative mycelial walls incubated with *V. fungicola* enzyme extract. **Figs 3, 4.** *A. bisporus* wall controls at 0 h and 72 respectively. **Figs 5–7.** Cell walls after 16, 24 and 72 h of incubation respectively with enzyme. Bars = 0·5 mm.

72 h of incubation and then decreased, except for the 1,3-β-glucanase which remained approximately the same. The production of other enzyme activities such as β-D-glucosidase, α-D-glucosidase, β-D-xylosidase, β-D-mannosidase, chitobiosidase, chitinase and protease was also determined after 72 h of incubation and their total and specific activities are shown in Table 1.




**Figs. 8–11.** Photographs of *V. fungicola* on right side and *A. bisporus* on left side in compost agar (**Fig. 8**) and Raper agar (**Fig. 11**) dual-cultures after 7, 11 and 15 d of incubation together. Light micrographs of the contact zone with intermingled hyphae of both fungi, in compost agar (**Fig. 9**) and Raper agar (**Fig. 10**).

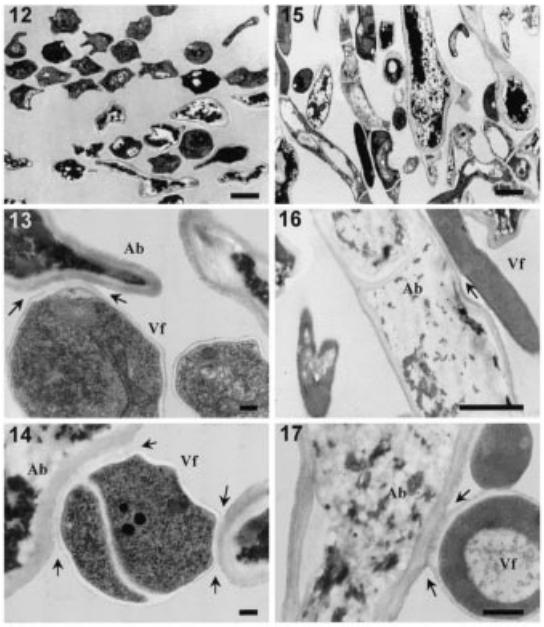
PAGE analysis of *V. fungicola* protein extract obtained in these conditions showed the presence of one major band (Fig. 2, lane 1) that corresponded to one of the three β-1,3-glucanase isozymes revealed by its specific test (Fig. 2, lane 2). Chitinase activities were also assayed on PAGE (Fig. 2, lane 3) and seven bands (A to G) were detected.

### *Lytic activity on purified cell walls*

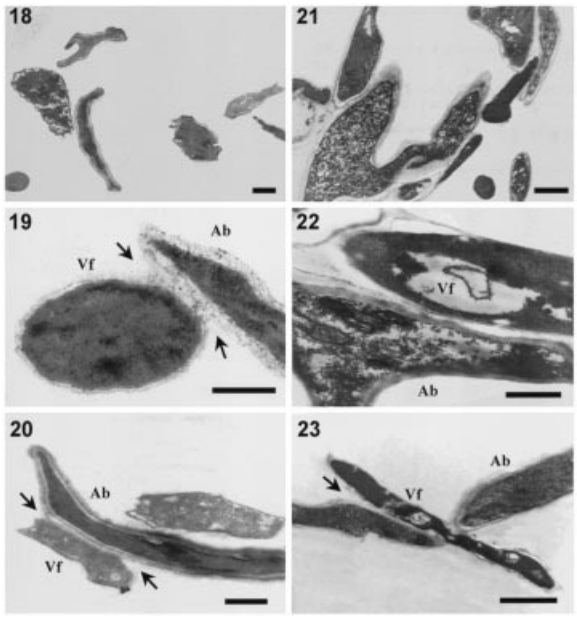
Figs 3–7 show the sequential degradation of isolated *A. bisporus* vegetative mycelial walls by the *V. fungicola* enzyme extract (Figs 5–7) together with the corresponding controls (Figs 3 and 4). The lytic effect of the enzyme solution could be seen after 16–24 h of incubation, though after 72 h the cell walls still had those components that permitted the maintenance of their shape. The gravimetric analysis of the cell walls after digestion showed a higher percentage of weight loss in the first 16 h (15·2%) and after the weight decreased less in the period to 24 h (20·3%) and 72 h (25·7%).

### *Hyphal interaction*

*V. fungicola* and *A. bisporus* in dual-culture grew toward each other and no visible inhibition zone was observed, although the growth reaction of both fungi was different, depending on the culture media used (Figs 8–11). Whereas in compost extract agar *A. bisporus* growth was unaffected and overgrew the hyphomycete (Fig. 8), in Raper agar *V. fungicola* growth was more abundant than in the other medium and grew until reaching *A. bisporus* (Fig. 11).




**Figs 12–17.** TEM micrographs of the interaction zone between *V. fungicola* and *A. bisporus* in Raper agar dual-culture 3 d after contact. **Figs 12–14.** Mainly transverse sections of the intermingled hyphae (bars = 1, 0·1 and 0·1 μm respectively). **Figs 15–17.** Mainly longitudinal sections of the interaction zone (bars = 1, 0·1 and 0·1 μm respectively). Arrows indicate the region of contact between the hyphae of both fungi. The hyphae of *V. fungicola* can be distinguished from those of *A. bisporus* by their smaller diameter, electron-dense contents of the cytoplasm and relatively thin cell wall, consisting of an electron-translucent layer in between the plasmalemma and outer layer.




**Figs 18–23.** TEM micrographs of the interaction zone between *V. fungicola* and *A. bisporus* in Compost agar dual-culture 3 d after contact. **Figs 18–20.** Mainly transverse sections of the intermingled hyphae (bars = 1, 0·5 and 1 μm respectively). **Figs 21–23.** Mainly longitudinal sections of the interaction zone (bars = 1, 0·5 and 1 μm respectively). Arrows indicate the point of contact between the hyphae of both fungi.

Under light microscopy, in individual and in paired-cultures, hyphae of *V. fungicola* could be readily distinguished from those of *A. bisporus* by their smaller diameter. Mycelial samples taken from the interaction zone of dual-cultures in compost and Raper agar 3 d after contact (Figs 9, 10) showed no evidence of alteration in either *A. bisporus* or *V. fungicola* hyphae.

In electron micrographs, the hyphae of *V. fungicola* were easily identified by their smaller diameters, electron-dense contents of the cytoplasm, and their distinct cell walls. Whereas in *V. fungicola* the relatively thin wall consisted of an electron-translucent layer in between the electron-dense inner and the outer layers, the wall of *A. bisporus* was seen as a thicker electron-homogeneous layer. Figs 12–17 show TEM micrographs of the interaction zone between *A. bisporus* and *V. fungicola* after 3 d of Raper agar dual-culture. Transverse and longitudinal sections showed that their hyphae intermingled. At the point of contact between the interacting hyphae, the *V. fungicola* appeared to be adhered to the *A. bisporus* surface though no symptoms of cell wall damage or cellular disintegration were observed in *A. bisporus* hyphae. The same effect was observed in compost agar culture medium (Figs 18–23).

The experiments carried out with the *A. bisporus* vegetative mycelium and the *V. fungicola* conidial suspension in the absence of culture medium showed that, although conidial germination occurred, there was no particular interaction between both organisms as there is in the presence of culture medium.

## DISCUSSION

In the present study the host-parasite interaction of *A. bisporus* and *V. fungicola* examined in paired-cultures with two different culture conditions showed that both growth reaction types were easily distinguishable. The investigation however has shown that these macroscopical differences exhibited by the distinct culture conditions are not reflected in microscopic differences of the individual hyphae.

Light microscopical examination of the contact zones revealed, on one hand, the *A. bisporus* overgrowing *V. fungicola* on compost extract, and on the other, the stopping of growth of both host and parasite mycelia upon contact in Raper medium. In both series of experiments apparent damage neither appeared in the corresponding host hyphae nor clearing halli of inhibition due to some antibiotic production by *A. bisporus*, as was suggested by Mamoun, Olivier & Vedie (1995). Electron microscopy showed the adhesion between the mycoparasite and its host, but there was no change in host cell wall degradation nor evidence of host penetration, as reported in the basidiome hyphae of the same organism (Dragt *et al.* 1996, Calonje *et al.* 1997). In this last case the presence of *A. bisporus* fruit body cell walls seemed to stimulate the overproduction of *V. fungicola* extracellular lytic enzymes giving rise to the degradation of the host cell walls and, at some particular sites, the obvious mycoparasite penetration inside the host cells. In the present work, however, the *A. bisporus* vegetative mycelium, after host-parasite contact, did not show any apparent changes.

Previous studies carried out by us (Calonje *et al*. 1997) have demonstrated that when *V. fungicola* was grown on *A. bisporus* fruit body, cell walls, produced an increased amount of carbohydrolases and proteases capable of digesting practically all the *in vitro* isolated fruit body walls. The same enzymes would be responsible for the *in vivo* fruit body cell wall degradation, although in this case the accessibility to the inner cell wall surface of the living cells is limited. In the present report similar studies carried out with *A. bisporus* vegetative mycelial walls, as the carbon source for *V. fungicola* growth, have shown that the mycoparasite produced practically the same carbohydrolases and proteases which also degraded *in vitro* isolated vegetative mycelial walls, although to a lesser extent on a microscopical basis, than in the case of isolated fruit body cell walls. One possible explanation of this finding may be the different chemical composition and structure of fruit body and vegetative mycelial walls (García Mendoza *et al*. 1987, Calonje, García Mendoza & Novaes-Ledieu 1996), which, *in vitro*, provide distinct resistance to *V. fungicola* enzyme complex.

Comparing the production of *V. fungicola* enzymes in the presence of either *A. bisporus* vegetative mycelial walls or fruit body walls as substrates, not only similarities, but also significant differences, can be encountered. The most striking finding is the sixfold higher content of 1,4-α-glucanase when fruit body cell walls were present in the culture medium (Calonje *et al*. 1997), although 1,4-β- and 1,3-α-glucanases also showed a slight increase in the same medium. This effect is difficult to interpret on the basis of the chemical differences of both kinds of *A. bisporus* walls employed, so the distinct enzyme activities produced by *V. fungicola* may be more related to its saprotrophic or parasitic behaviour.

The fact that *V. fungicola* does not seem to exhibit any lytic effect on *A. bisporus* vegetative mycelial walls *in vivo* may be due to some inhibition of either the enzyme production or the corresponding enzyme activity. Another hypothesis suggested by Manocha, Chen & Rao (1990) in other mycoparasitism cases is that, after the initial recognition and attachment, the mycoparasite probably has to recognise differences between the compatible and the incompatible host hyphae possibly at the plasma membrane level, without excluding changes in the physiology of both fungi induced by environmental factors.

These observations suggest that the resistance of *A. bisporus* vegetative mycelium to *V. fungicola* mycoparasitism could be due to several factors in which the architecture of the cell wall plays an important role. Investigation is now in progress on the chemical composition and structure of *V. fungicola* cell walls in an attempt to demonstrate their possible specific attachment to the *A. bisporus* fruit body walls as the first step of the infection process.

## ACKNOWLEDGEMENTS

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