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# Suppression of *Verticillium* wilt in eggplant by some fungal root endophytes

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## Abstract

One hundred and twenty-three fungal isolates were obtained from 225 root segments of eggplants, melon, tomato, strawberry and Chinese cabbage, grown as bait plants in a mixed soil made up of samples from different fields in Shizuoka, Japan. Isolates belonging to Mycelium radicis atrovirens (MRA), including *Phialocephala fortinii*, were the most prevalent in all the five bait plants. Eleven of the 123 isolates, after being inoculated onto axenically reared eggplant seedlings, almost completely suppressed the pathogenic effects of a post-inoculated, virulent strain of *Verticillium dahliae*. Seven of these 11 isolates had come from the roots of eggplant and included *Heteroconium chaetospira, P. fortinii,* and unidentified species of *Fusarium, Penicillium*, *Trichoderma* and MRA. *P. fortinii*, *H. chaetospira*, a non-sporulating isolate with white mycelium (SWM) and MRA were easily reisolated from root segments. Hyphae of *H. chaetospira, P. fortinii* and SWM colonized the root tissues of eggplant without causing apparent pathogenic symptoms. The mechanisms by which these endophytes confer resistance to infection by *V. dahliae* are unknown but the effectiveness of these fungi in a laboratory setting indicates that they have potential as biocontrol agents and merit further investigation.

## Introduction

*Verticillium* wilt, caused by the soilborne fungus, *Verticillium dahliae* Kleb., is one of the most destructive disease of eggplant (Bletsos et al., 1999). Control of the pathogen has relied on chemical control agents such as methyl bromide (Jarvis, 1993), but following The Montreal Protocol of 1991, the manufacture and trade of methyl bromide will be phased out in 2005.

A reduction in the use of the chemical agents could be achieved through the development of biological methods. Several studies have reported the control of *Verticillium* wilt in eggplant using biocontrol microorganisms (Marois et al., 1982; De Melo et al., 1987; Turhan, 1981; Yamaguchi et al., 1992). However, biocontrol agents for the disease have not been accepted widely by growers. These microorganisms, i.e., *Talaromyces flavus, Trichoderma hamatum, T. lignorum, T. viride* and *Streptomyces ochraceiscleroticus*, inhabit the rhizosphere rather than the root cortical tissues and are affected by an array of competitive interactions with other microorganisms.

However, microorganisms that are able to colonize the rhizosphere and then enter, survive or proliferate endophytically can be efficient biocontrol agents (Tjamos, 2000). Narisawa et al. (2000) found that the root endophytic hyphomycete *Heteroconium chaetospira* suppressed *Verticillium* yellows in Chinese cabbage in the field. Based on this observation, and the fact that the roots of plants in their natural habitats are usually colonized by apparently harmless endophytic fungi (Jumpponen and Trappe 1998), we proposed the hypothesis that naturally occurring fungal root endophytes have the potential to suppress disease in their host plants. To test this hypothesis, a suite of root endophytic fungi from soil was obtained using common herbaceous crop species as bait plants. Axenically reared eggplant seedlings were inoculated with these

endophytes, one at a time, and then post-inoculated with a spore suspension of *V. dahliae*. Here, these procedures are described and the features or identity of the isolates that were effective in suppressing *Verticillium* wilt in eggplant seedlings are presented. In addition, the appearance of hyphae of some endophytic isolates within eggplant roots is described.

## Materials and methods

### *Isolation of root endophytic fungi*

Soil samples were taken in September 1996 from four fields each of which had been used to grow organic crops of Chinese cabbage (*Brassica campestris* L.), tea (*Camellia sinensis* L. (O. Kuntze)), corn (*Zea mays* L.) and white clover (*Trifolium repens* L.) in Shizuoka, Japan. From each field, five soil samples (approximately 1 l each) were taken from the area around the roots, placed in polyethylene bags and stored at 4 °C for up to 5 months. Soil samples were combined and mixed to prepare a composite soil for baiting fungal endophytes.

To bait for root endophytic fungi, axenically grown seedlings of eggplant (*Solanum melongena* L.), melon (*Cucumis melo* L.), tomato (*Lycopersicon esculentum* Mill., nom. cons.), strawberry (*Fragaria grandiflora* J.F. Ehrh.) and Chinese cabbage were transplanted into 200 ml pots containing the composite soil. There were three seedlings in each pot, and three replicate pots for each plant species were made. After two months, the roots of each plant species from each series of replicate pots were cleaned and cut into 1 cm segments. Forty-five segments from each species were chosen at random for isolating endophytes according to the methods described in Narisawa et al. (1998). Briefly, this involved washing root segments in a 0.005% solution of Tween 20 three times and rinsing each sample three times in distilled water. Segments were air dried overnight and then placed on nutrient agar. Fungal isolates were identified based on morphology of sporulating structures or, in the case of non-sporulating fungi, grouped according to colony color.

### *Inoculation of eggplant with fungal isolates and disease assessment in pot-grown plants*

The *Verticillium* wilt-susceptible eggplant (*S. melongena* L.) cv. 'Senryou 2-go' (Takii seed Co., Kyoto, Japan) was used for disease assessment. Seedlings were challenged with the fungal endophytes as follows. Each isolate was grown on corn meal malt yeast extract (CMMY) medium (25 g corn meal (infusion form) (Difco), 15 g Bacto agar (Difco), 10 g malt extract (Difco), 2 g yeast extract (Difco) per liter) in Petri dishes for several days at room temperature. Two mycelial plugs (approximately 5 $mm^2$) were placed on autoclaved (30 min) peat pellets (40 mm diameter, Jiffy-7, AS Jiffy Products Ltd., Norway) which had been soaked in malt yeast (ME) medium (malt extract 10 $gl^{-1}$ and yeast extract 2 $gl^{-1}$). The inoculated peat pellets were then incubated at 25 °C in the dark. One month later, when hyphae of the inoculant was evident on the surface, a single 5-day-old eggplant seedling grown in gnotobiotic conditions was transplanted into each peat pellet. Eggplant seedlings transplanted in non-inoculated, soaked peat pellets were used as controls. Seedlings were incubated at 20–25 °C under a 16 h photoperiod for 1 month.

To prepare inoculum of the pathogen, *V. dahliae* (No. 84011, National Agriculture Research Center, Japan) was grown on potato dextrose agar (PDA, Difco) in Petri dishes for 1 week at room temperature. Three mycelial plugs (approximately 5 $mm^2$) were placed in each of three 300 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB, Difco). The flasks were cultured statically for 1 week at room temperature. Fungal mycelium was separated from the broth by filtering the culture through eight layers of sterile gauze. The mycelium remaining on the gauze was washed three times with sterile water. The mycelium from each of the 3 flasks was combined and macerated in a homogenizer with 180 ml sterile deionized water. The macerate was centrifuged at 1700 g for 10 min using a swinging bucket rotor. To wash the macerate, it was resuspended in 180 ml sterile deionized water and centrifuged again. This process was repeated three times. The resulting suspension of conidia and hyphal segments was diluted with sterile deionized water to $1 \times 10^7$ propagules per ml using a hemacytometer.

Seedlings grown for 1 month in the presence of individual endophytes or not (control) were challenged with the pathogen. The suspension of *V. dahliae* propagules was poured into a tray containing the seedlings in pellets and held for 2 h at room temperature (approximately 23 °C). The seedlings were then transplanted to 1 l plastic pots containing a commercial non-sterile soil mix (Sakata Seed Co., Yokohama, Japan), and grown in a greenhouse at 20–25 °C. Disease

symptoms were assessed 3 weeks after transplanting, using the disease index of Narisawa et al. (2000) with slight modifications. External symptoms were visually rated among four classes; 0 (no foliar symptoms), 1 (yellowing of leaves), 2 (wilt of leaves) and 3 (death of all leaves and the apical bud). The number of plants in class 1 was multiplied by 10, in class 2 by 60, and in class 3 by 100 and the sum including the number of plants in class 0, was divided by the total number of plants in each treatment to give a numerical value or 'the disease index'. Nine plants in each treatment were recovered from 3 pots and symptoms of the disease were noted. The experiment was replicated three times.

*Reisolation of fungal isolates from root tissues*

After wilt symptoms were assessed, sets of seedlings with a disease index below 3 were selected for the reisolation of the inoculant. Inoculated fungi were reisolated from roots (Narisawa et al., 1998) by washing with Tween 20 and sterile water, followed by drying overnight. Segments were placed on nutrient agar. Fifteen root segments were chosen randomly from each run. The frequency of reisolation was calculated as the mean number of root segments colonized by the fungus. Analysis of variance was performed using the Abacus Concept, StatView procedure (Abacus Concept, Inc., Berkeley, CA). The frequency of reisolation data was analyzed for significance after arcsin transformations using analysis of variance followed by Fisher's protected least significant difference (PLSD) test. Root segments, up to 3 cm in length and drawn from the same set of roots used for reisolation, were stained with 0.005% cotton blue in 50% acetic acid, and observed under an Olympus BX50 microscope with UPlanFI40/0.75 and UPlanFI100/1.30 objectives to assess colonization levels.

## Results

One hundred and twenty-three isolates were obtained from a total of 225 root segments of eggplant, melon, tomato, strawberry and Chinese cabbage. Eggplant root segments yielded the greatest number of fungi with isolates obtained from 58% of segments. From Chinese cabbage roots, 45% of the segments yielded fungi, while from tomato, melon and strawberry roots, 31 to 33% of segments yielded fungi. Isolates belonging to Mycelium radicis atrovirens (MRA), including *Phialocephala fortinii*, were the most prevalent in all the five species, with the greatest number of the isolates (18 isolates, 40%) from eggplant. Other taxa were recovered in much smaller numbers from all crops (Table 1). Unidentified species of *Cladosporium, Fusarium, Mortierella, Paecilomyces, Penicillium, Pythium, Rhizoctonia and Trichoderma* were mostly isolated within 5 days of placing root segments on the medium. *H. chaetospira*, MRA (including *P. fortinii*) and a sterile white mycelium (SWM) were mostly isolated after 1–3 weeks.

*Table 1*. Frequency of fungal taxa isolated from root segments of herbaceous bait plants

| Fungal taxon | Total and relative (%)[a] number of isolates | | | | |
|---|---|---|---|---|---|
| | Eggplant | Tomato | Melon | Strawberry | Chinese cabbage |
| *Cladosporium* spp. | 2 (4.4) | 0 (0) | 2 (4.4) | 0 (0) | 2 (4.4) |
| *Fusarium* ssp. | 2 (4.4) | 3 (6.6) | 4 (8.8) | 2 (4.4) | 2 (4.4) |
| *H. chaetospira* | 5 (11) | 3 (6.6) | 0 (0) | 0 (0) | 0 (0) |
| *Mortierella* sp. | 0 (0) | 1 (2.2) | 0 (0) | 0 (0) | 0 (0) |
| MRA[b] | 18 (40) | 10 (22) | 10 (22) | 14 (31) | 12 (27) |
| *Paecilomyces* ssp. | 1 (2.2) | 0 (0) | 0 (0) | 2 (4.4) | 2 (4.4) |
| *Penicillium* ssp. | 4 (8.8) | 0 (0) | 0 (0) | 0 (0) | 4 (8.8) |
| *Pythium* ssp. | 0 (0) | 0 (0) | 2 (4.4) | 0 (0) | 0 (0) |
| *Rhizoctonia* ssp. | 0 (0) | 0 (0) | 0 (0) | 1 (2.2) | 0 (0) |
| SWM[c] | 3 (6.6) | 1 (2.2) | 0 (0) | 2 (4.4) | 4 (8.8) |
| *Trichoderma* ssp. | 0 (0) | 0 (0) | 0 (0) | 0 (0) | 4 (8.8) |
| Total number of root segments yielding fungi | 26 (58) | 14 (31) | 14 (31) | 15 (33) | 18 (45) |

[a]Number of root segments colonized by the fungus/total number of segments × 100, $n = 3$.
[b]Mycelium radicis atrovirens, including *P. fortinii*.
[c]Non-sporulating fungus with white mycelium.

*Table 2*. Effects of fungal isolates from bait plants on the incidence of *Verticillium* wilt and their frequency of reisolation from inoculated eggplant at the conclusion of the experiment

| Taxon (isolate number)[a] | Disease index[b] | Bait plant | Frequency of reisolation (%)[b,f] |
|---|---|---|---|
| *Fusarium* sp. 1 (MTB1) | 0 | Tomato | 13[a] |
| *Fusarium* sp. 2 (MNS1) | 0 | Eggplant | 13[a] |
| *Fusarium* sp. 3 (MNB3) | 0 | Eggplant | 7[a] |
| MRA[c]1 (MTJ1) | 0 | Tomato | 20[a] |
| MRA 2 (MIB3) | 0 | Strawberry | 33[b] |
| MRA 3 (MNB9) | 0 | Eggplant | 7[a] |
| *Penicillium* sp. (MNT8) | 0 | Eggplant | 13[a] |
| SWM[d] (MHB2) | 0 | Chinese cabbage | 33[b] |
| *H. chaetospira* (MNB4) | 3 | Eggplant | 33[b] |
| *P. fortinii* (MNJ1) | 3 | Eggplant | 67[c] |
| *Trichoderma* sp. (MNS11) | 3 | Eggplant | 13[a] |
| Control[e] | 100 | — | — |

[a]Only fungal isolates depressing the disease index to 3 or lower are listed.
[b]See Materials and methods for method of calculation.
[c]MRA = Mycelium radicis atrovirens.
[d]Non-sporulating fungus with white mycelium.
[e]Uninoculated with fungal isolates but challenged with *V. dahliae*.
[f]Values with the same letter are not significantly at $P = 0.05$, Fisher's PLSD test, $n = 3$.

*Effect of isolated fungi on disease incidence*

Eggplants that had been pre-inoculated with eight of the fungal root endophyte isolates did not show symptoms of *Verticillium* wilt and plants inoculated with three isolates showed only slight yellowing of the leaves (Table 2). Internal symptoms, such as vascular discoloration of the root or hypocotyl, were not observed on these seedlings. Plants inoculated with all other isolates were slightly to extremely wilted. Both xylem and adjacent tissues of the plants were dark brown. The average index of external symptoms of these plants was 72.8. The percentage of plants with a disease index of 3–20 and over 20 was 3% and 86%, respectively. Seven of the 11 effective isolates, were originally isolated from eggplant and included one isolate of MRA, two of *Fusarium* species, one of *H. chaetospira*, one of *Penicillium* species, one of *P. fortinii*, *Trichoderma* species. One MRA and one *Fusarium* species originated from tomato, one MRA from strawberry and one SWM from Chinese cabbage. No effective isolates were obtained from melon (Table 2).

*Growth of root endophytes in root tissues of eggplant*

The 11 effective isolates from roots showing no severe symptoms of *Verticillium* wilt were reisolated from the root segments, cultured on CMMY medium, and identified as the original inoculum. *P. fortinii* (MNJ1) grew from a large number (67%) of the root segments. *H. chaetospira* (MNB4), SWM (MHB2) and MRA (MIB3) were also easily recovered, growing from 33% root segments ($P = 0.05$) (Table 2). The pathogen, *V. dahliae* was not recovered from these root segments (data not shown). Hyphae of *H. chaetospira* (MNB4) and *P. fortinii* (MNJ1) from eggplant, and SWM (MHB2) from Chinese cabbage also extensively colonized the root of eggplant without causing visible external symptoms. Hyphae of the other effective isolates, belonging to the genera *Fusarium, Penicillium* and *Trichoderma*, may have colonized the root surface or possibly been present in the rhizosphere. Intracellular hyphae of *H. chaetospira* occurred in cortical cells of the root axis and extended to root tips (Figure 1a). Hyphae of *P. fortinii* grew along the surface of the root, and formed black sclerotia on/in the epidermal layer (Figure 1b). Hyphae of the SWM heavily colonized some root cells of the cortex (Figure 1c). In all cases, morphology of these inoculants in roots was distinctive and compatible with our previous studies of these species in root tissues. They all exhibited unique differences in colonization morphology, i.e. *P. fortinii* produced dematiaceous, lobed hyphae and sclerotic cells, *H. chaetospira* produced smooth dematiaceous hyphae especially in the vicinity of root tips, and SWM formed dense masses of hyaline hyphae inside root

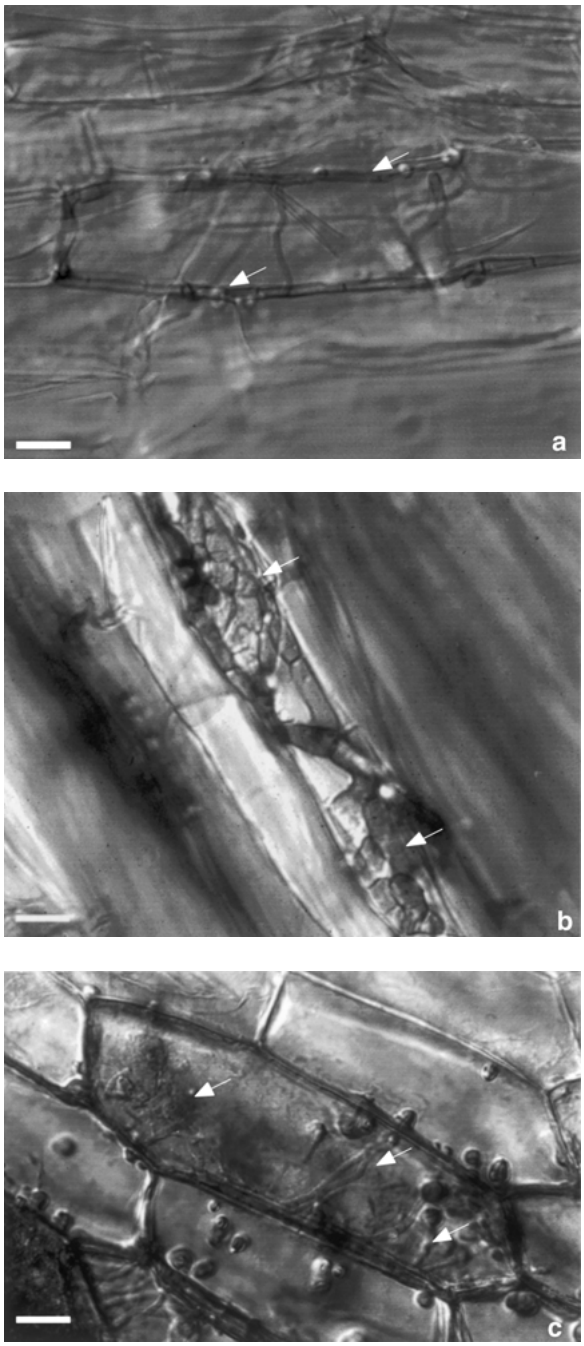


*Figure 1*. (a) Hyphae (arrow) of *H. chaetospira* (MNB4) produced smooth dematiaceous hyphae on/in root tissue of eggplant. (b) *P. fortinii* (MNJ1) formed dematiaceous, lobed hyphae and sclerotic cells (arrows) on/in cortical cells of eggplant root. (c) Dense masses of hyaline hyphae (arrows) of sterile white mycelia (MHB2) colonized cortical cells of eggplant root. Root segments were stained with 0.005% cotton blue in 50% acetic acid, and observed with a photomic microscope (bars, 10 μm).

cells. Details of the colonization pattern of these fungi in roots were not studied.

## Discussion

Over half of the isolates obtained were identified as *P. fortinii*, a dematiaceous hyphomycete in the MRA complex and a common and widespread endophyte from the roots of many plant species and different habitats (Addy et al., 2000; Jumpponen and Trappe, 1998). Currah et al. (1993) suggested that the high frequency of recovery of *P. fortinii* might be related to its habit of forming resistant microsclerotia in the cells of host plant root. It is apparently quickly invasive and has little or no visible effects on the host plant, while its effect on dry weight accumulation is negligible, slightly negative or even positive (Fernando and Currah, 1996). The ecological significance of *P. fortinii* therefore is unclear, although it has been suggested that the symbiosis may be tolerated by the host plant because of antagonistic effects of the endophyte toward arthropod grazers, or because of its ability to function as an allelopathic agent against competing plants, or because of a putative mycorrhizal role (Jumpponen and Trappe, 1998; Fernando and Currah, 1996; O'Dell et al., 1993). A role in the suppression of the effects of root pathogens has not been demonstrated prior to this work. Isolates of *H. chaetospira* were less frequent than *P. fortinii*, representing 7% of the fungi baited out of the composite soil. This species has been reported from roots (Narisawa et al., 1998), arable soils (Domsch et al., 1993), as well as from the wood of deciduous trees, and from millipede droppings (Matsushima, 1975; Ellis, 1976). It has been shown to suppress the development of *Plasmodiophora brassicae*, *V. dahliae* in Chinese cabbage (Narisawa et al., 2000).

There was some striking variation among plant species in their effectiveness as baits. Eggplant was the most effective bait species. The greatest number of fungi with isolates obtained from eggplant root compared to other crops. Among these, a large number of dark fungi were obtained. MRA was the most prevalent (18 isolates, 40%). *H. chaetospira* was the second most prevalent (5 isolates, 11%). Eggplant was the most effective bait for the isolation of the root endophytic fungi from this composite field soil and under cultural conditions used. More trials with different soils and under different conditions would indicate if eggplant is more predisposed towards colonization than other plants.

Biocontrol methods could target a number of events in the developmental sequence of the pathogen. For example, microsclerotia are important structures involved in the dissemination and induction of disease by *V. dahliae* and the ascomycete, *T. flavus* has been shown to reduce the fitness of this pathogen by parasitizing these resting bodies (Tjamos, 2000). Alternatively, other types of antagonism, or interference with the pathogen's ability to enter host tissue, could be a target mechanism in the development of biocontrol agents. In this study, we selected 11 isolates that had the ability to suppress *Verticillium* wilt. The mode of suppression by the isolates of *Fusarium, Trichoderma* and *Penicillium* is unknown, but it is suspected that they directly antagonize *V. dahliae* in the rhizosphere (Tjamos, 2000). Species of *Fusarium, Trichoderma* have been shown to suppress *Verticillium* wilt in eggplant by previous workers (Yamaguchi et al., 1992; De Melo et al., 1987). It is possible that the endophytes (*H. chaetospira*, SWM and *P. fortinii*) function to suppress *Verticillium* wilt by inducing a systemic resistance in the host plant. Endophytic fungi are known to induce (and survive) strong antagonistic responses in host plants (Schulz et al., 1999) and these may be sufficient to provide resistance to pathogens that otherwise can invade plants without initiating a strong defense response. Narisawa et al. (2000) reported that *H. chaetospira* could inhibit the development of clubroot and *Verticillium* yellows in Chinese cabbage. *H. chaetospira* did not parasitize microsclerotia of the fungus nor produce any inhibition zones in dual cultures with the pathogens (unpublished data).

Even though *P. fortinii* and *H. chaetospira* can colonize a variety of host plants (Haselwandter and Read, 1982; Currah et al., 1987; Stoyke and Currah, 1991; Jumpponen and Trappe, 1998; Narisawa et al., 2000), our preliminary data indicate that disease suppression could be, at least in part, strain specific because most of the isolates effective in suppressing *Verticillium* wilt in eggplant were isolated originally from eggplant. Narisawa et al. (1998) also showed that the suppression of clubroot disease and *Verticillium* yellows in Chinese cabbage was most effective with isolates that had been obtained from Chinese cabbage.

*V. dahliae* enters its host through cells in the root tip elongation zone (Huisman and Gerik, 1989), and consequently fungi selected to serve as antagonists to this organism should be able to colonize this zone. Intracellular hyphae of *H. chaetospira*, *P. fortinii* and SWM were well developed in cortical cells including root tip region and tended to form sclerotia or sclerotium-like structures in roots. *P. fortinii* grew from a large number of root segments when these were placed on agar media for reisolating the inoculant. *H. chaetospira*, SWM and other isolate of MRA were also easily recovered. Narisawa et al. (2000) reported that *H. chaetospira* could, when growing as a root endophyte, inhibit the development of clubroot and *Verticillium* yellows in a field setting. The ability of these fungi to gain entry into the root tissues seems integral to their ability to suppress the ingress of the pathogen, at least when propagules of the pathogen are spores and hyphal segments.

Given the above preliminary observations, we are encouraged to accept the hypothesis that there are naturally occurring root endophytic fungi that can suppress disease in host plants, at least under artificial conditions. In nature, quite different results might be expected if the invasive cells of the pathogen originate from other diseased tissues, or from other propagules such as microsclerotia. How the fungal endophytes confer resistance to a pathogen is yet to be determined.

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