Evaluation of *Fusarium* as potential biological control against *Orobanche* on Faba bean in Tunisia

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**Summary**

A total of 149 fungal strains identified as *Fusarium* were isolated from infected *Orobanche crenata* Forsk. and *Orobanche foetida* Poir. plants. Their pathogenicity and virulence were assessed in Petri dish assays using lentils as the medium. Ten isolates were found to reduce the number of tubercles attached to the host plant. Among them, two isolates that caused necroses on tubercles of *Orobanche* in the Petri dish assays were identified as *Fusarium F6* and *F10*. They reduced the number of tubercles of *O. crenata* by 97% and 98%, respectively. Inoculums of *F6* and *F10* were produced on barley grains and were tested in sterilized and non-sterilized soil in separate pot experiments, using *O. crenata* and *O. foetida* as parasitic plants. Both isolates reduced the number of *O. crenata* and *O. foetida* by 68% and 88%, respectively, and their dry matter by 82% to 88%. A similar experiment conducted using formulated inoculums of the two isolates showed that the formulation improved the efficiency of the fungi, and reductions in the number and dry matter of tubercles to 100% were observed. These results suggest that *Fusarium* isolates have the potential to be used as biological control agents against *O. crenata* and *O. foetida* on faba bean in Tunisia.

**Keywords**: pathogens, pathogenicity, virulence, parasitic plants, broomrapes.

**Introduction**

Broomrapes, *Orobanche* spp., of the family of *Orobanchaceae* are troublesome root parasitic weeds that cause severe damage to vegetables, legumes and sunflower (Parker and Riches, 1992). Approximately, 16 million hectares of arable land in the Mediterranean region as well as in west Asia are currently endangered by *Orobanche* infestation (Sauerborn, 1994). In Tunisia, *Orobanche crenata* Forsk. distributed in the north-east and *O. foetida* Poir. in the north-west, are the main species that cause losses in leguminous crops, especially on faba bean (Kharrat and Hallila, 1994). Losses in faba bean fields can reach 80% (Kharrat, 2002). Difficulties in controlling *Orobanche* are due to the numerous tiny seeds that retain their viability in the soil for 6 to 20 years. Germination of *Orobanche* seed requires a stimulant excreted by the host plant and produces germ tubes that attach to the host plant (Raynal et al., 1989). The germ tube develops a haustorium and forms a tubercle. The haustorium represents the physical and morphological contact between the parasite and the host. It supplies the parasite with water, mineral nutrients and organic materials from its host (Kroschel, 2001).

So far, no efficient control measures for *Orobanche* spp. have become available to farmers (Müller-Stöver, 2001). Single methods such as delayed sowing and use of resistant varieties have shown unsatisfactory results. The use of chemical products such as glyphosate requires care to avoid phytotoxicity. Thus, *Orobanche* represents a difficult target for selective chemical control. Control of *Orobanche* may be possible by integrating control measures. The integration of biological control with other *Orobanche* management methods is of increasing research interest. Several investigators have reported the use of fungi as potential biological agents against *Orobanche* (Wastson and Waymore, 1992).
Research has been conducted in several countries including Algeria, Egypt, Germany, Morocco and Chili (Klein et al., 1999; Zermane et al., 1999; Müller-Stöver, 2001; Boari and Vurro, 2004). *Fusarium oxysporum* f. sp. *orthoceras* (Appel and Wollenw.) Bilai obtained from diseased *O. cumana* tested in soil with sunflower as a host plant was able to reduce the number of attached and emerged broomrape seedlings by about 90% (Bedi and Donchev, 1991). *F. oxysporum* f. sp. *orthoceras* on *O. cumana* was also tested by Thomas et al. (1998). Recently, *F. arthroporoides* and *F. oxysporum* isolated in Israel from *O. aegyptiaca* were shown to be effective in reducing broomrape growth (Amsellem et al., 2001).

The pathogenicity of two isolates, *Ulocladium botrytis* Preuss and *F. oxysporum* Schlecht. f. sp. *Orthoceras*, were tested by Müller-Stöver (2001). The two fungi cause necroses on both *O. cumana* and *O. crenata*. Currently, the development of an appropriate formulation which allows successful application of fungal propagules will determine the success of *Fusarium* in agriculture applications. The encapsulation of fungal propagules in a solid matrix ‘Pesta’ was used by (Müller-Stöver, 2001). A 70% reduction of *Orobanche* emergence was obtained when wheat flour kaolin granules containing chlamydospore rich biomass was applied.

Considering the importance of *O. crenata* and *O. foetida* in Tunisia and the lack of research on fungi associated to *Orobanche* spp., the main objective of this study was to screen and evaluate the potential of fungi isolated from *Orobanche* with potential as biological control agents against the parasitic weed, in laboratory and greenhouse experiments.

**Materials and methods**

**Field surveys**

Field surveys were carried out from April 2004 to May 2005 in northern Tunisia, especially in the region of Nabeul. Underground stages of *Orobanche* with symptoms of fungal infections such as browning and rotting were collected. The plants were conserved in laboratory until use.

**Isolation**

Isolations were made from pieces of tubercles and stems with fungal symptoms. Diseased tissues were excised, washed with distilled water, sterilized in 1% sodium hypochlorite with Tween 20 for 5 min and rinsed four to five times with sterile distilled water. After drying on filter paper, pieces were placed in Petri dishes on potato dextrose agar (PDA™) medium supplemented with 100 ppm of streptomycin. The Petri dishes were incubated in the dark at 22°C until fungal development occurred. Repeated sub-culturing was done to obtain pure cultures. Isolates were conserved on special nutrient poor agar (SNA) at 5°C for short term storage and in liquid nitrogen for long-term storage.

**Bioassays**

The isolated fungi were evaluated to assess their phytotoxic ability on the growth of the underground stages of *Orobanche*. In these Petri dishes bioassays, seeds of *O. crenata* were used as the parasitic weed and those of lentil were used as the host plant. The methods followed those of Kroschel (2001). Plastic Petri dishes were filled with washed sterile sand, watered and covered with filter paper. *Orobanche crenata* seeds were sterilized with sodium hypochlorite, rinsed with distilled water and sprinkled on the filter paper at the densities of 25 seeds per square metre. The Petri dishes were covered with black plastic and incubated in the dark for conditioning at 22°C for 10 days. To enhance pre-conditioning in the *Orobanche* seed, 100 ppm of gibberellic acid was added. Pre-germinated lentil seedlings were inserted into sand in the Petri dishes through holes made in the surface of the filter paper.

To test the pathogenicity of fungal isolates, fresh colonies of the isolated fungi growing on SNA medium were used. For each Petri dish, the black plastic was removed and filter paper containing *O. crenata* seeds and lentil seedlings were sprayed with 10 ml of the spore suspension at 10⁶ spores per millilitre. The Petri dishes were incubated in the greenhouse at 25°C and 16 h/18 h photoperiod for 5 weeks. Two replicates were used per treatment. The number of germinated, attached seeds and the number of tubercles formation were recorded.

**Test of specificity**

The isolates which reduced the number of tubercles of *Orobanche* in Petri-dish assays were selected and were tested for their host specificity. A range of plants was used that included tomato (*Lycopersicon esculentum* Mill), carrot (*Daucus carota* L.), Faba bean (*Vicia faba* L. and *Vicia faba* L. minor), pea (*Pisum sativum* L.), chickpea (*Cicer arietinum* L.), lentil (*Lens culinaris* L.), wheat (*Triticum aestivum* L.) and barley (*Hordeum vulgare* L.). These plants were grown in pots in a greenhouse. Ten days after emergence roots were washed with distilled water and immersed in the inoculums at the concentration of 10⁶ spores per millilitre, for 5 to 10 min. Then plants were transplanted in pots and observed weekly for 1 month for the development of symptoms. Four plants of the same test plant species were used per pot with four pots per tested plant.

**Pot assays**

Selected fungal isolates that were able to reduce *Orobanche* seed germination in the Petri dish assays were tested in sterilized and non-sterilized soils in pots in a greenhouse. Both *O. crenata* and *O. foetida* were used as parasitic weeds and *V. faba* as the host plant during this experiment.
Preparation of inoculum: Barely grains were used as a solid inoculum. Ten ml of sterile water were added to fresh colonies of the fungi growing on SNA medium. The resulting suspension, containing spores and mycelium, was used to inoculate the organic substrate which was then incubated for 14 days at 25°C.

Inoculation and pot trials: Two pot experiments, using sterilized and non sterilized soils, were conducted under greenhouse conditions to study the ability of fungi to control O. crenata and O. foetida in V. faba plants. In both experiments, plastic pots with 750 g capacity were filled with a mixture of soil and sand at a ratio of 2:1. Orobanche seeds were sprinkled onto the soil surface. The inoculum was added and mixed into the soil together with the seeds. Each pot was provided with either 11 mg of O. crenata seed or 9 mg of O. foetida seed, or 7.5 g of the solid inoculum. Three seeds of V. Faba minor were planted per pot and thinned afterwards to obtain one host plant per pot. The pots were fertilized and watered each week. Five controls were prepared: the host plant alone (H), the host plant with O. crenata or host plant with O. foetida, the host plant with non-inoculated substrate (H + NIS) and host plant with inoculated substrate (H + NIS). The experiments continued for 5 months and terminated when the host plant in the control was dead. The parameters used to assess the effect of fungi on the control of Orobanche were faba bean height (cm) and dry matter weight (g) and Orobanche number and dry matter weight (g).

Formulation in pot experiments

Wheat flour kaolin granules were prepared after the methods of Connik et al. (1991, 1996). Durum wheat flour 32 g, kaolin 6 g and sucrose 2 g, were blended and poured into a dish. Inoculums were added as chlamydomspore in 23 ml PDB (potato dextrose broth). The mixture was kneaded with gloved hands and passed through a small, hand-operated pasta maker. Obtained granules were incorporated in sterilized and non-sterilized soils. The granules were added and mixed into the soil together with the seeds. Each pot was filled with either 11 mg of O. crenata or 9 mg of O. foetida and 1 g of granules per kg of soil. Three seeds of V. faba minor were planted per pot and thinned afterwards to obtain one host plant per pot. In total, five pot experiments were carried out.

Statistical analysis

All pots experiments were conducted in totally randomized design. Statistical analyses were performed using analysis of variance (ANOVA) with alpha 0.05 in GEN-STAT software.

Results

Field survey and isolation of fungi

One hundred and forty nine isolates were obtained from infected O. crenata plants collected during field surveys. All isolates were found to belong to the genus Fusarium after microscope examination.

Bioassays

Ten isolates of the genus Fusarium reduced the germination of O. crenata 27% to 93% and eight reduced attachment (22% to 79%) to the host plant by germinated Orobanche (Table 1). The two exceptions, F4 and F7, did not differ statistically between the treatments and the non-inoculated control. The number of tubercles developed by O. crenata was reduced by 78% to 98% (Table 1). Symptoms of necroses were observed on Orobanche inoculated tubercles.

Isolates F6 and F10 were the most efficient in reducing the percentage of tubercles of O. crenata by

Table 1. Effect of a conidial suspension of Fusarium on the development of the underground stages of Orobanche crenata in Petri dishes including; the number of O. crenata seeds that germinated, the number of germinating seeds that attached to the host and the number of tubercles formed.

<table>
<thead>
<tr>
<th>Isolates</th>
<th>Number germinated</th>
<th>Percent reduction in germination</th>
<th>Number attached</th>
<th>Percent reduction in attachments</th>
<th>Number of tubercles</th>
<th>Percent reduction in tubercles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>36.5</td>
<td>–</td>
<td>27</td>
<td>–</td>
<td>47</td>
<td>–</td>
</tr>
<tr>
<td>F1</td>
<td>7.5</td>
<td>79</td>
<td>5.5</td>
<td>79</td>
<td>7.5</td>
<td>84</td>
</tr>
<tr>
<td>F2</td>
<td>3.5</td>
<td>90</td>
<td>18</td>
<td>33</td>
<td>10</td>
<td>78</td>
</tr>
<tr>
<td>F3</td>
<td>5</td>
<td>86</td>
<td>10.5</td>
<td>61</td>
<td>2</td>
<td>95</td>
</tr>
<tr>
<td>F4</td>
<td>3</td>
<td>92</td>
<td>29</td>
<td>–</td>
<td>7.5</td>
<td>84</td>
</tr>
<tr>
<td>F5</td>
<td>5</td>
<td>86</td>
<td>9</td>
<td>67</td>
<td>5</td>
<td>89</td>
</tr>
<tr>
<td>F6</td>
<td>26.5</td>
<td>27</td>
<td>13.5</td>
<td>50</td>
<td>1</td>
<td>98</td>
</tr>
<tr>
<td>F7</td>
<td>3.5</td>
<td>90</td>
<td>24.5</td>
<td>9</td>
<td>4.5</td>
<td>90</td>
</tr>
<tr>
<td>F8</td>
<td>4</td>
<td>89</td>
<td>13</td>
<td>52</td>
<td>4</td>
<td>91</td>
</tr>
<tr>
<td>F9</td>
<td>2.5</td>
<td>93</td>
<td>13.5</td>
<td>50</td>
<td>7</td>
<td>85</td>
</tr>
<tr>
<td>F10</td>
<td>23</td>
<td>37</td>
<td>21.5</td>
<td>22</td>
<td>1.5</td>
<td>97</td>
</tr>
<tr>
<td>LSD(5%)</td>
<td>5.74</td>
<td>–</td>
<td>4.03</td>
<td>–</td>
<td>3.5</td>
<td>–</td>
</tr>
</tbody>
</table>
97% and 98% respectively. These isolates were used in sterilized and non sterilized soil on *O. crenata* and *O. foetida* using faba bean as a host plant.

**Test of specificity**

Isolates *F6* and *F10* were selected for use in specificity test because in preliminary tests no symptoms and no death were observed on test plant species.

**Pot assays**

**Sterilized soil:** *F6* and *F10* tested in sterilized soil on *O. crenata* and *O. foetida* reduced the number germinated and dry matter weights of both parasitic plants. In *O. crenata* the number of tubercles was reduced by 70% to 87% compared to the infested the controls (*H + O + NIS*), and the dry matter of tubercles was reduced by 88% (Table 2). Inoculation with *F6* and *F10* isolates resulted in 36% to 38% increase in height of faba bean compared to the *Orobanche* infested control (*H + IS*). The dry weight of the host plant was also significantly increased by 120% to 129% compared to the *Orobanche*-infected control (Table 2).

Isolates *F6* and *F10* reduced the number of *O. foetida* by 68% to 77% whereas the dry matter was reduced by 81% to 84% compared to the infested control (*H + O + NIS*) (Table 2). The height and the dry matter of Faba bean was also increased by 35% to 45% and 79% to 82%, respectively, compared to the infested control (*H + IS*).

**Non-sterilized soils:** Isolates *F6* and *F10* incorporated with barley grains as inoculum substrate into the soil reduced the number of both *O. crenata* and *O. foetida* by more than 90% compared to the infested *Orobanche* control (Table 3). So, the dry matter of both *O. crenata* and *O. foetida* was increased by 100%, which did not statistically differ from both control of *O. crenata* and *O. foetida* (*H + O + NIS*) (Table 3).

There were no significant effects on the height or dry matter of faba bean compared to either *Orobanche* species (Table 3). High significantly, no tubercles were produced by either species.

**Formulation experiments in pots**

There were no tubercles produced and therefore no tubercle dry matter for *O. crenata* and *O. foetida* when wheat flour kaolin granules containing chlamydospore rich biomass was applied in sterilized or non sterilized soils (Figure 1).

**Discussion**

The use of Petri dishes allowed observation of the underground stages of *Orobanche* (germination, attachments and tubercles) which would not have been possible

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**Table 2.** Effect of isolates *F6* and *F10* on the number of tubercles and tubercle dry weight of *Orobanche crenata* and *O. foetida* and on the height and dry weight of faba bean, in sterilized soil.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant height (cm)</th>
<th>Percent increase</th>
<th>Plant dry matter (g)</th>
<th>Percent increase</th>
<th>No. of tubercles</th>
<th>Percent reduction</th>
<th>Tubercles dry matter (g)</th>
<th>Percent reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (<em>H</em>)</td>
<td>64.8</td>
<td>–</td>
<td>5.39</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (<em>H + IS</em>)</td>
<td>65.4</td>
<td>–</td>
<td>5.65</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (<em>H + F6</em>)</td>
<td>89.4</td>
<td>37</td>
<td>9.41</td>
<td>66</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (<em>H + F10</em>)</td>
<td>89</td>
<td>36</td>
<td>8.6</td>
<td>52</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (<em>H + O</em>)</td>
<td>51.4</td>
<td>–</td>
<td>2.66</td>
<td>–</td>
<td>5.2</td>
<td>2.02</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (<em>H + O + NIS</em>)</td>
<td>53.8</td>
<td>–</td>
<td>2.77</td>
<td>–</td>
<td>4.8</td>
<td>1.93</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>(<em>F6</em>)</td>
<td>73</td>
<td>36</td>
<td>6.11</td>
<td>120</td>
<td>0.6</td>
<td>87</td>
<td>0.23</td>
<td>88</td>
</tr>
<tr>
<td>(<em>F10</em>)</td>
<td>74.2</td>
<td>38</td>
<td>6.35</td>
<td>129</td>
<td>1.4</td>
<td>70</td>
<td>0.25</td>
<td>88</td>
</tr>
<tr>
<td>LSD (5%)</td>
<td>11.33</td>
<td>–</td>
<td>1.81</td>
<td>–</td>
<td>2.18</td>
<td>1.14</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 2.** Effect of isolates *F6* and *F10* on the number of tubercles and tubercle dry weight of *Orobanche crenata* and *O. foetida* and on the height and dry weight of faba bean, in sterilized soil.

**Table 2.** Effect of isolates *F6* and *F10* on the number of tubercles and tubercle dry weight of *Orobanche crenata* and *O. foetida* and on the height and dry weight of faba bean, in sterilized soil.

**Table 2.** Effect of isolates *F6* and *F10* on the number of tubercles and tubercle dry weight of *Orobanche crenata* and *O. foetida* and on the height and dry weight of faba bean, in sterilized soil.
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Table 3. Effect of isolates F6 and F10 on the number of tubercles and tubercle dry weight of Orobanche crenata and Orobanche foetida and on the height and dry weight of Faba bean, in non sterilized soil.

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Plant height (cm)</th>
<th>Plant dry weight (g)</th>
<th>No. of tubercle</th>
<th>Tubercles dry weight (g)</th>
<th>Plant height increase</th>
<th>Plant dry weight increase</th>
<th>Percent reduction</th>
<th>Percent reduction</th>
<th>Vica faba minor, faba bean</th>
<th>Orobanche crenata</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (H)</td>
<td>56.4</td>
<td>1.9</td>
<td>2</td>
<td>0.025</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (H + IS)</td>
<td>49.8</td>
<td>1.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (H + F6)</td>
<td>64</td>
<td>1</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (H + F10)</td>
<td>51.8</td>
<td>1.2</td>
<td>2</td>
<td>0.025</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (H + O)</td>
<td>39.8</td>
<td>0.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Control (H + O + NIS)</td>
<td>37.6</td>
<td>0.9</td>
<td>2.2</td>
<td>0.025</td>
<td>0</td>
<td>100</td>
<td>0</td>
<td>0</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td>(F6)</td>
<td>45.6</td>
<td>1.9</td>
<td>111</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
</tbody>
</table>
| (F10)               | 45.4              | 1.6                  | 77              | –                        | –                     | –                       | –                 | –                 | –                        | –                 | NS
| LSD (5%)            | NS                | NS                  | 0.59            | NS                      | NS                    | –                       | –                 | –                 | –                        | –                 |

H Faba bean only; H + NIS fava bean plus non-inoculated barley grains; H + O fava bean plus Orobanche; H + O + F fava bean plus non inoculated barley grains plus Orobanche.

Figure 1. Pesta formulation effect on the number of Orobanche crenata and O. foetida tubercles.

The percentage of attachment of O. crenata to the lentil host plant was also reduced by 79% as a result of fungus inoculum. Bedi and Donchev (1991) suggested that the black pigment of seeds protects the seed from fungal attack and they believed that the infection by the fungus occurred after seed germination. Consequently, the number of tubercles formation was significantly reduced by 98% in Petri dishes assays. Müller-Stöver (2001) found that the mortality of O. aegyptiaca tu-
bercles was significantly increased after inoculation in a root chamber. Accordingly, the same phenomenon were also observed by Bouzoukov and Kouzmanova (1994). Thomas et al. (1998) suggested that F. oxysporum inoculated in a root chamber, attacked germination and tubercles formation. Cohen et al. (2002) explained the mortality process of tubercles; they suggested that the hyphen penetrated the outer cells layer within 24 h, reaching the centre of the tubercles by 48 h and infected nearly all cells by 72 h. Most of the infected tubercles had died by 96 h. We observed necroses on inoculated tubercles and the same were observed by Linke et al. (1992).

Of the ten isolates tested, two F6 and F10 were the most effective in reducing the percentage of tubercles in Petri dishes. Tests in with these two isolates in sterilized and non-sterilized soil showed that they could significantly reduce the number and dry weight of O. crenata and O. foetida. Similarly, Sauerborn et al. (1994) found a reduction in tubercle number of O. cumana parasitizing sunflower. Müller-Stöver (2001) observed a decrease of the total O. cumana dry matter per pot as a consequence of the application of fungi. The same phenomena were also observed by Thomas et al. (1998) for O. cumana and F. oxysporum f. sp. orthoceras.

Faba bean height and dry weight increased when F6 and F10 were used on barley grains as inoculum substrate. Zonno and Vurro (2002) using F. oxysporum and F. solani on O. ramosa, with tomato as the host plant, suggested that both isolates permitted growth of a larger and healthier tomato root system compared to their controls. The same reduction in numbers and dry weights of O. crenata and O. foetida were observed by us in non-sterilized soil.

These results suggested that Fusarium was able to grow and compete successfully with other microorganisms present in the soil (Abbasher et al., 1996). The use of Fusarium to control O. crenata and O. foetida in soil has not previously been considered for biological control. The potential of mycoherbicides for use against the parasitic plants has been investigated (Garcia Garza et al., 1998) and our studies indicate that this may be possible using Fusarium to control Orobanche species. Future research will be done to identify isolate F6 and F10 using the morphological and molecular technique.

**References**


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