Bulgarian Chemical Communications, Volume 49, Special Edition K2, (pp. 5 – 10) 2017

Screening of an attenuated strain of *Fusarium oxysporum* f. sp. cucumerinum using rhizosphere soil treated with ethanol extract of parsley (*Apium graveolens*)

Q. Lijin [1, 2], S. Zhiyong[1], G. Cheng[1], Q. Cheng[1], Y. Xingfu[2]

[1] *College of Life Science, Chifeng University, Chifeng 024000, China;*
[2] *College of Agronomy, Inner Mongolia Agricultural University, Hohhot 010019, China*

Received, August 1, 2017; Accepted, November 21, 2017

In the present study, an ethanol extract of parsley (*Apium graveolens*) fresh rhizosphere soil was supplemented to PDA plates for successive culturing of *Fusarium oxysporum* f. sp. cucumerinum, to screen an attenuated strain of *F. oxysporum* f. sp. *cucumerinum*. The results revealed that 50 mg·mL^{-1} ethanol extract of parsley fresh rhizosphere soil had allelopathic inhibitory effect on the first to fifth generation of *F. oxysporum* f. sp. *cucumerinum*. The diameter of colonies displayed significant difference between ethanol extract-treated and control 48 and 144 h after inoculation (P <0.05). *F. oxysporum* f. sp. *cucumerinum* from the first to fifth generation was attenuated in virulence by the ethanol extract of parsley rhizosphere soil, and the virulence of *F. oxysporum* f. sp. *cucumerinum* decreased with the increase in generation number. As a result, an attenuated strain of *F. oxysporum* f. sp. *cucumerinum* with only 6.7% virulence was screened out from the fifth generation.

Key words: Parsley (*Apium graveolens*), Rhizosphere soil, Ethanol extract, Fusarium wilt of cucumber, Attenuated strain

INTRODUCTION

Fusarium wilt is an important disease in cucumber and melons, which generally causes 10% to 30% loss in cucumber yield, even up to 80% to 90% in some cases, leading to huge economic losses. In recent years, Fusarium wilt has become increasingly prevalent in cucumber due to the intensive and continuous cropping system, and thus has caused great economic losses to farmers. Fusarium wilt as a common vascular wilt fungal disease, is difficult to control with chemicals in the field. Although large amounts of agricultural chemicals have been used to control the disease, Fusarium wilt remains a serious threat to cucumber, and the abuse of agricultural chemicals has resulted in serious pollution to cucumber products and environment [1]

Currently, grafting between cucumber and pumpkin is the most effective way to prevent Fusarium wilt in cucumber. However, due to the difficulties in grafting technology and the cumbersome management procedures after grafting, the survival rate of grafted seedlings is very low, and the cucumber quality is also obviously declined by grafting, so this technology cannot be popularized widely. As Fusarium wilt is still a major problem in the production of cucumber, it is urgent to screen attenuated strains (biological "vaccine") of *Fusarium oxysporum* f. sp. *cucumerinum* to control Fusarium wilt in rhizosphere soil containing ethanol extract of parsley for several generations, to induce abnormal division, growth and development of this pathogen, which was then inoculated into cucumber seedlings to detect the virulence of every generation, till an attenuated strain of *F. oxysporum* f. sp. *cucumerinum* was screened out. It is expected that the attenuated strain can be used instead of agricultural chemicals to control Fusarium wilt in cucumber. The results may provide a theoretical basis for the production and development of pollution-free, green and safe vegetables.

EXPERIMENTAL

Materials

American four-season parsley was provided by Chunfeng Vegetable Breeding Farm in Qing County of Hebei Province. The cucumber variety Jinchun No. 4 was developed by Tianjin Kerun Agricultural Science and Technology Co., Ltd. *F. oxysporium* f. sp. *cucumerinum* strain was purchased from the Laboratory of Plant Pathology, Institute of Vegetables and Flowers, Chinese Academy of Agricultural Sciences.

Parsley seeds were germinated by soaking in water before they were sown in a greenhouse on January 24, 2016. Then, 60-day seedlings were transplanted into a greenhouse at the Scientific Research Base of Inner Mongolia Agricultural University on March 24, 2016, under conventional field managements.

Fresh parsley roots were excavated from 10 random sites, and 20 g of soil was collected from every site. The soil samples were bulked and packed in a plastic bag. In laboratory, 60 g of the soil was weighed, divided into three portions of

*To whom all correspondence should be sent:
E-mail: yxf5807@163.com

equal weight in brown jars. After absolute ethanol (analytical grade) was added, the jars were placed on a reciprocating shaker at 25ºC and 240 r·min^{-1} for 24 h [1]. Then, the raw ethanol extract was filtered first with qualitative filter paper, and then with 0.22 μm bacterial filter. The resulting mother liquid of the ethanol extract of parsley rhizosphere soil was 200 mg·mL^{-1}.

Methods

Measurement of the allelopathic effects of the ethanol extract of parsley rhizosphere soil on F. oxysporum f. sp. cucumerinum.

The mother liquid of the ethanol extract of parsley rhizosphere soil was diluted 4 times to the working concentration of 50 mg·mL^{-1}. Then, 2 mL of the diluted ethanol extract was added into 18 mL molten PDA medium to make plates (ethanol extract treatment), while in ethanol control (CK) the diluted ethanol extract was replaced by 2 ml of 25% ethanol, and nothing was added to normal PDA plates in negative control.

F. oxysporium f. sp. *cucumerinum* discs (0.6 cm diameter) were made using a hole puncher, and inoculated into the center of the agar plates prepared as described above, with the mycelium side facing down and one disc in each plate. Plates were cultured at 25 °C. The colony diameter in each treatment was measured once every 24 h, from 48 to 144 h after inoculation (when the fungal mycelia of the control covered the whole plate). Five replicates were set for each treatment, and the mean colony diameter of the five replicates was used to measure allelopathic effects [1]. Actual colony diameter (cm) = mean colony diameter measured -0.6.

Measurement of the virulence of F. oxysporum f. sp. cucumerinum treated by the ethanol extract of parsley rhizosphere soil

The conidia were washed with sterile water off the plates that had been cultured for 144 h and collected. The cell concentration of the suspension was calculated using a hemocytometer under a microscope, and diluted to 1.0 × 10^6 cells·mL^{-1} before inoculation. After disinfection and germination, the cucumber seeds with radicles of 1 cm long were soaked in the above conidia suspension for 20 min, and then sown into plastic pots filled with high-pressure sterilized soil, covered with small sheds and plastic films to maintain heat and moisture. When 60% of the seedlings emerged, the plastic films were removed. Three replicates were prepared for each treatment, with 20 seedlings in each replicate.

The disease index of each treatment was surveyed once every other day from disease occurrence till the disease incidence in control stabilized. The disease classification and identification were performed by referring to Weng [2], as follows:

Level 0: no obvious wilt symptoms; level 1: cotyledons turned yellow but did not wilt; level 2: cotyledons completely wilted or the seedlings slightly wilted; level 3: seedlings obviously wilted or dwarfed; level 4: seedlings wilted and died (or did not emerge). Disease index = Σ (disease level × the number of seedlings at this level) /the highest disease level × total number of seedlings × 100%.

Screening of attenuated strains of F. oxysporum f. sp. cucumerinum during successive subculturing

The colonies that had been cultured for 144 h according to "*Measurement of the allelopathic effects of the ethanol extract of parsley rhizosphere soil on F. oxysporum f. sp. cucumerinum*" were considered as the first generation. The discs of the first-generation colonies of ethanol extract treatment, ethanol control and negative control were made using a hole puncher, inoculated into the center of fresh PDA plates, respectively, cultured in dark in an incubator for 144 h to yield the second-generation colonies. By the same way, further generations of colonies were cultured. Attenuated strains of *F. oxysporum* f. sp. *cucumerinum* in each generation were screened following the steps described in "*Measurement of the allelopathic effects of the ethanol extract of parsley rhizosphere soil on F. oxysporum f. sp. cucumerinum*" and "*Measurement of the virulence of F. oxysporum f. sp. cucumerinum treated by the ethanol extract of parsley rhizosphere soil*".

Data processing and plotting were performed with Excel 2007, data analysis and Duncan's multiple comparison were carried out with SPSS 17.0.

RESULTS AND DISCUSSION

The allelopathic effects of the ethanol extract of parsley rhizosphere soil on the first to the fifth generation of *F. oxysporum* f. sp. *cucumerinum* are shown in Figures 1, 2. 48 h after inoculation, the colony diameter of the first to fifth generation in ethanol extract treatment was respectively by 76.7%, 66.5%, 72.5%, 67.1% and 67.2% less than that in ethanol control, indicating that the colony growth was inhibited by the allelopathic effect of the ethanol extract of parsley rhizosphere soil. 144 h after inoculation, the colony diameter in ethanol extract treatment was respectively by 40.5%, 22.0%, 47.0%, 38.0%, and 13.8% less than that in ethanol control, and the colony growth was still inhibited by the allelopathic effect of the ethanol extract. There was significant difference in colony diameter between ethanol extract treatment and ethanol control from 48 to 144 h after inoculation (P <0.05).

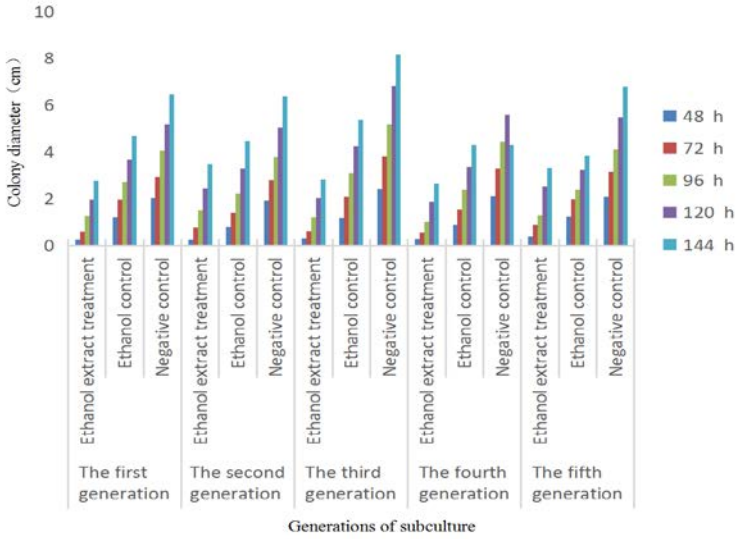

Fig. 1. Allelopathic effect of the ethanol extract of parsley rhizosphere soil on the five generations of *F. oxysporum* f. sp. *cucumerinum* during successive subculturing

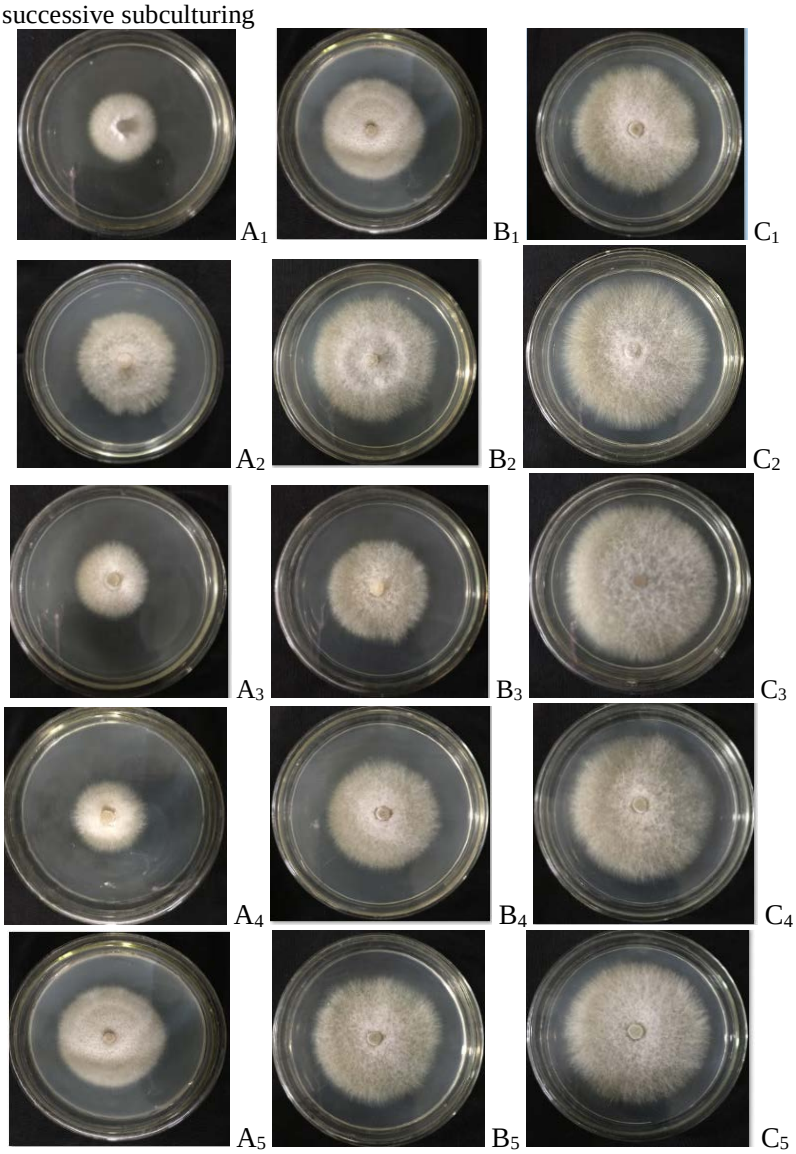

Fig. 2. Colony growth of the first to the fifth generation of *F. oxysporum* f. sp. *cucumerinum* 144 h after inoculation. $A_{1\text{-}5}$, Ethanol extract treatment; $B_{1\text{-}5}$, Ethanol control; $C_{1\text{-}5}$, Negative control

Changes in the virulence of *F. oxysporum* f. sp. *cucumerinum* treated by the ethanol extract of parsley rhizosphere soil during successive subculturing.

The virulence of first-generation *F. oxysporum* f. sp. *cucumerinum* was different between the treatments (Fig. 3). Seven days after inoculation, the disease index in ethanol extract treatment was 11.7, which was by 33.1% lower than that in ethanol control (17.5). Fifteen days after inoculation, the disease index in ethanol extract treatment was 23.3, which was by 22.3% lower than that in ethanol control (30). The fact that the virulence of *F. oxysporum* f. sp. *cucumerinum* was lowered by the ethanol extract suggested that the ethanol extract of parsley rhizosphere soil was capable of attenuating the virulence of *F. oxysporum* f. sp. *cucumerinum*. There was significant difference in the virulence of *F. oxysporum* f. sp. *cucumerinum* between ethanol extract treatment and ethanol control 7, 11, 13, and 15 days after inoculation ($P < 0.05$).

As shown in Fig. 3, 7 days after inoculation, the disease index in ethanol extract treatment was 6.7, which was by 33% lower than that in ethanol control (10), indicating that the virulence of *F. oxysporum* f. sp. *cucumerinum* was attenuated by ethanol extract. Fifteen days after inoculation, the disease index in ethanol extract treatment was 17.5, which was 30% lower than that in ethanol control (25.0), suggesting that the virulence of *F. oxysporum* f. sp. *cucumerinum* was still attenuated by he ethanol extract. The second generation of *F. oxysporum* f. sp. *cucumerinum* showed lower virulence than the first generation 15 days after inoculation, indicating that the attenuating effect of the ethanol extract on the pathogen increased from the first generation to the second generation. There was significant difference in the virulence of *F. oxysporum* f. sp. *cucumerinum* between ethanol extract treatment and ethanol control 13 and 15 days after inoculation ($P < 0.05$).

As shown in Fig. 3, 7 days after inoculation, the disease index in ethanol extract treatment was 0, while that in ethanol control was 10, indicating that the virulence of *F. oxysporum* f. sp. *cucumerinum* was attenuated by the ethanol extract from the third generation to the fifth generation. Fifteen days after inoculation, the disease index in ethanol extract treatment was 15, which was by 35.6% lower than that in ethanol control (23.3), indicating that the virulence of *F. oxysporum* f. sp. *cucumerinum* was still attenuated by ethanol extract. The third generation of *F. oxysporum* f. sp. *cucumerinum* had smaller disease index than the second generation, suggesting that the attenuating effect of the ethanol extract increased from the second generation to the third generation.

As shown in Fig. 3, fifteen days after inoculation, the disease index in ethanol extract treatment was 11.7, which was by 41.5% lower than that in ethanol control (20), indicating that the virulence of *F. oxysporum* f. sp. *cucumerinum* was continuously attenuated by the ethanol extract. Compared with the third generation, the disease index of fourth-generation *F. oxysporum* f. sp. *cucumerinum* treated by ethanol extract decreased to 11.7, so an attenuated strain of *F. oxysporum* f. sp. *cucumerinum* could be screened out by further treatment.

As shown in Fig. 3, fifteen days after inoculation, the disease index in the ethanol extract treatment was 6.7, which was by 66.5% lower than that in ethanol control (20), indicating that the virulence of *F. oxysporum* f. sp. *cucumerinum* was continuously attenuated by the ethanol extract. Inaddition, the disease index of the fifth generation decreased from 11.7 in the fourth generation, to 6.7 indicating that the virulence of *F. oxysporum* f. sp. *cucumerinum* was gradually attenuated by ethanol extract treatment during successive subculturing. An attenuated strain with a disease index of 6.7 was screened out in the fifth generation.

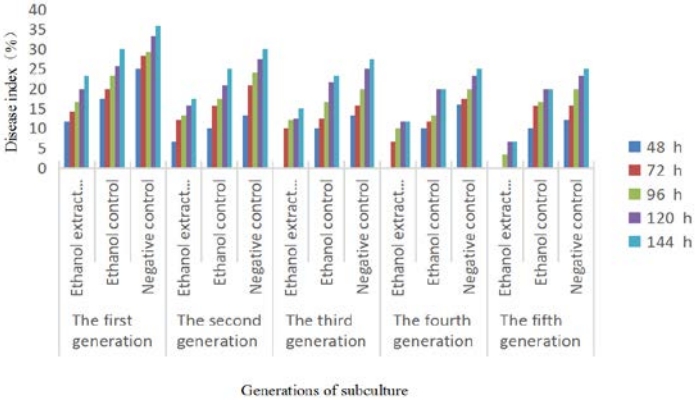

Fig. 3. Changes in the virulence of the five generations of *F. oxysporum* f. sp. *cucumerinum* during successive subculturing

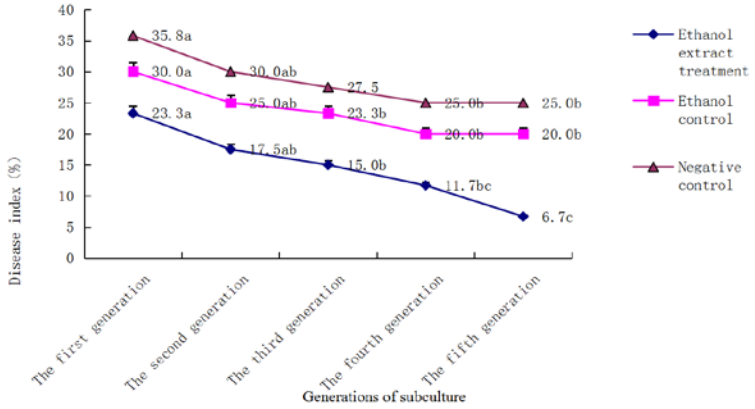

Fig.4. Intergenerational changes of the virulence of *F. oxysporum* f. sp. *cucumerinum* during successive subculturing

Intergenerational changes of the virulence of F. oxysporum f. sp. cucumerinum

In general, the virulence of *F. oxysporum* f. sp. *cucumerinum* gradually decreased from the first to the fifth generation during successive subculturing. In ethanol extract treatment, the maximum disease index (23.3) appeared in the first generation, and was lower than that in the controls of the same generation. The minimum disease index (6.7) appeared in the fifth generation, which was lower than that in the controls of the same generation. The virulence of *F. oxysporum* f. sp. *cucumerinum* in all treatments gradually increased from the first to the fifth generation. But the range of intergenerational decrease was different, indicating different attenuating effects between these treatments. In ethanol extract treatment, there was no significant difference in the disease index between two adjacent generations, but significant difference between skipped generations (P <0.05). In addition, the virulence of the negative control decreased at first and then became stable with the increase in generation number, indicating that the virulence of *F. oxysporum* f. sp. *cucumerinum* was gradually attenuated during successive subculturing (Fig. 4).

CONCLUSIONS

Allelopathy refers to the beneficial or detrimental effects of the metabolic exudates of one plant or microorganism on another plant or microorganism in the environment. The existing studies of allelopathy are mainly focused on the effects of allelopathic plants on the receptor plants, the allelopathic autotoxicity of plants, the separation of allelopathic components, the identification of allelopathic plants and their action mechanism on microorganisms [3-5].The allelopathic effect of plant extracts on pathogens is a hot issue in recent studies, which not only reveals the mechanism of continuous cropping obstacles of vegetable crops, but also opens up a new way to prevent and control crop diseases.

The allelopathic effects of the extracts of parsley fresh roots, rotten roots and seeds on *F. oxysporum* f. sp. *cucumerinum* and related mechanisms have been studied in our earlier works Reference 6-8. Based on this, *F. oxysporum* f. sp. *Cucumerinum* was treated with 50 mg·mL^{-1} ethanol extract of parsley fresh rhizosphere soil for five successive generations in the present study to explore the allelopathic effect of the ethanol extract on the first to fifth generations of *F. oxysporum* f. sp. *cucumerinum*. The results showed that 50 mg·mL^{-1} ethanol extract inhibited the growth of all five generations of *F. oxysporum* f. sp. *cucumerinum*, which validated that the ethanol extract of parsley rhizosphere soil contained active allelopathic substances that inhibited the growth of microorganisms.

F. oxysporum has formae specialis (f. sp.) that infect a variety of hosts causing various diseases, physiological races, and several non-pathogenic strains. Such strains can be obtained by natural screening or artificial mutagenesis, and are used to control wilt in crops. Wu and Wang isolated two attenuated strains with 4.35% and 7.14 virulence from disease cucumber seedlings, and proved that attenuated strains of *F. oxysporum* f. sp. *cucumerinum* could induce resistance of cucumber to wilt [9]. In the study of Gu *et al*., a non-pathogenic mutant of *F. oxysporum* f. sp. *cucumerinum* was isolated by UV mutagenesis and proved to be positive in wilt prevention by artificial inoculation [10]. A non-pathogenic *F. oxysporum* strain was isolated by UV mutagenesis from cucumber and melon by Yang *et al*. All the results suggested that attenuated strains of *F. oxysporum* were mostly isolated by physical methods, but rarely using the allelopathic effects of plants on microorganisms.

The results of the present study showed that the ethanol extract from parsley rhizosphere soil had allelopathic inhibitory effects on all the five

generations of *F. oxysporum* f. sp. *cucumerinum* during successive subculturing, and the virulence of *F. oxysporum* f. sp. *cucumerinum* was gradually attenuated from the first to the fifth generation in all treatments. An attenuated strain of *F. oxysporum* f. sp. *cucumerinum* with 6.7% virulence was screened out by ethanol extract treatment from the fifth generation.

Acknowledgements: *This work was financially supported by the Natural Science Foundation of China (31160100) and the Applied Technology Research and Development Project of Inner Mongolia Autonomous Region (20150711).*

REFERENCES

1. C. Qian, X.F. Yun, X.M. Gao, Y.Y. Bao, D.D. Zhang, J. Li, Y.Wang, *Chinese Journal of Eco-Agriculture*, **21**(05), 606 (2013).
2. Z.X. Weng, X.X. Jiang, X.W. Xiao, *China Vegetables*, **2**, 30 (1985).
3. H. Molish, *Fischer*, 13, 1937.
4. B.S. Singh *New Bot.*, **9**, 13 (1982).
5. E.L. Rice, Allelopathy (2nd ed.), New York: Academic Press, 1, 1984.
6. Y. Cao, The study on the allelopathy of extracts of the rotten root and the rhizosphere zone rot soil of parsley on *Fusarium oxysporum* sp. *Cucumeris*, Inner Mongolia Agricultural University, Hohhot, 2009.
7. J.Y. Jia, Study of allelopathy mechanism of extracts of parsley seed on *Fusarium oxysporium* f. sp. *Cucumeris*, Hohhot: Inner Mongolia Agricultural University, 2011.
8. X.M. Gao, Study on the isolating and identificating allelochemicals in the extract solution of parsley and its' induced resistance on the cucumber wilt, Inner Mongolia Agricultural University, Hohhot, 2015.
9. Y.C. Wu, S.Z. Wang, *Journal of Henan Agricultural University*, **4**, 433 (1991).
10. Z.M Gu, J. Chen, Z.G. Gao, J.H. Zhuang, *World Pesticides*, **2**, 46 (2007).