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Exploring a soil fumigation strategy based on ammonium bicarbonate to control *Fusarium* wilts of cucurbits

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ABSTRACT

Fusarium wilt diseases, caused by formae speciales of the fungus *Fusarium oxysporum* are a serious problem for the production of cucumber, watermelon, melon and other plants around the world. In this study, the possibility of applying a novel fumigation agent to the soil was investigated as a strategy for controlling *F. oxysporum* f. sp. *cucumerinum* (FOC), *F. oxysporum* f. sp. *niveum* (FON), and *F. oxysporum* f. sp. *melonis* (FOM). The fumigation agent ammonium bicarbonate, which releases ammonia, was investigated when applied alone and with lime as a direct soil amendment under sealed conditions. For the FOC, FON and FOM, the suitable additive concentrations of ammonium bicarbonate were 2, 2 and 1.5 g kg^{-1} soil dry weight (DW), respectively, when applied alone. These concentrations produced the best effects when short processing times, broad temperature ranges and wide soil moisture contents were used. Ammonium bicarbonate had a stronger antifungal effect when mixed with lime than when applied alone in the pot experiments. In the field experiment, stronger antifungal effects were observed when the pH value of the soil was less than 7, and no significant difference between the two treatments was observed in alkaline soil. This study explored a novel fumigation agent for controlling *F. oxysporum* that was based on ammonium bicarbonate and provided a potential strategy for ensuring healthy cucumber, watermelon and melon crops and supporting the worldwide development of these cropping industries.

1. Introduction

Fusarium oxysporum is an important soil-borne plant pathogen and is widely distributed in various soil types worldwide (Fravel et al., 2003). Pathogen formae speciales infect more than one hundred plant species, including cucumber, watermelon and melon caused by *F. oxysporum* f. sp. *cucumerinum* (FOC), *F. oxysporum* f. sp. *niveum* (FON) and *F. oxysporum* f. sp. *melonis* (FOM), respectively. Furthermore, *F. oxysporum* is reported as the most limiting factor for the worldwide production of these plants (Chen et al., 2010; Ling et al., 2011; Zhao et al., 2011). In addition, these pathogens can survive by chlamydospores in the soil for many years or live as a saprophyte by colonizing dead organic matter under varied environmental conditions (Agrios, 2005). The high capacity for survival may be due to these pathogens could colonize roots of rotation crops which limits the effectiveness of crop rotation (Katan, 1971; Scott et al., 2014). The ability of these pathogens to colonize crops on which they do not cause disease is well documented, for example, *F. oxysporum* f. sp. *lactucae* can colonize roots of cauliflower, broccoli and spinach without causing disease symptoms (Scott et al., 2014). Thus, control of *Fusarium* wilt is more difficult in fields (Nel et al., 2006). Breeding cultivars that are resistant to *Fusarium* wilt, grafting, and soil solarization are among the leading methods for controlling *Fusarium* wilts (Omar et al., 2006; Wang et al., 2013). However, new virulent strains of *F. oxysporum* species will appear along with commercialization of resistant varieties (Wang et al., 2013). The soil solarization (Tamietti and Valentino, 2006) is often limited by local climate constraints. Biocontrol is also an alternative, however, its efficiency is always influenced by abiotic and biotic factors (Fuchs et al., 1999; Jiménez-Díaz et al., 2011).

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Soil pretreatment, such as soil fumigation with methyl bromide, methyl iodide, propargyl bromide, calcium cyanamide or 1, 3-dichloropropene, has been widely used to control weeds, soil-borne fungi, insects, plant parasitic nematodes, and other disease causing organisms in high-value agricultural production systems (Luo et al., 2010). However, due to the potential environmental damage by many of these chemicals, the use of fumigant pesticides is restricted (Dong and Zhang, 2006; Wesemael et al., 2011). Similarly, calcium cyanamide is no longer available because of the environmental health and phytotoxicity risks of the intermediate anion CN^{2-} (Humpherson-Jones et al., 1992; Williamson and Dyce, 1989). In addition, various physical methods, including steam disinfection, soil solarization and hot water injection, have been employed with varying success to control nematodes as an alternative to soil fumigation with synthetic chemicals (Decker, 1989). However, many factors, including the soil type, climatic conditions and the soil water content, can affect the effectiveness of physical treatments (Dungan et al., 2003). Thus, it is important to explore new soil fumigants and their application methods to ensure healthy crops and to maintain the health of the environment.

Several reports have demonstrated that ammonia-releasing materials, both organic and inorganic, have utility as nematicides/fungicides (Oka et al., 2003; Bashour et al., 2013) through soil injection (Eno et al., 1955; Smiley et al., 1970). In addition, aqua ammonia (NH_4OH, 28% N) has been reported to control nematodes and Fusarium and Verticillium wilt in tomato plants grown in infested soil plots in greenhouse experiments in Lebanon (Bashour et al., 2013). In soils that were treated with compounds at a concentration of 200 mg N/kg, the aqua ammonia and NH_4HCO_3 resulted in similar fumigation effects (Oka and Pivonia, 2002). However, although many papers regarding the antifungal activity of ammonia and ammonia production materials have been published in the last 40 years, the use of ammonia and ammonia production materials for controlling soil-borne diseases has not been accepted commercially because the nematicidal and antifungal activities of ammonia depend on environmental conditions, especially soil pH, which is the most important factor that affects the nematicidal activity of ammonia (Rodriguez-Kabana et al., 1987). In acid conditions, the free ammonia (NH_3) in the water is ionized to form ammonium ions (NH_4^+), which are not nematicidal (Du Plessis and Kroontje, 1964). In recent years, the use of ammonia and ammonia production materials for controlling fungal diseases has been reconsidered in China because methyl bromide and other chemical compounds will be withdrawn from the market in the near future due to its deleterious effects on the ozone layer (Dong and Zhang, 2006). However, to our knowledge, no reports have described the effects of anhydrous ammonia and ammonia production materials when used as fumigants for different formae speciales of *F. oxysporum*.

In acid soils, a marked decrease in acidity should occur in the soils that are amended with free lime to adjust the soil pH. The fact that ammonia-releasing materials combined with lime applied as a soil fumigant could increase alkalinity of the soil has led to an interest in its value as an antifungal agent (Tsao and Oster, 1981; Rousk et al., 2009). In this study, ammonium bicarbonate applied alone and combined with lime were used as soil fumigants to explore an environmentally friendly, safe, and long-lasting method for controlling *F. oxysporum* in acid and alkaline soils.

2. Materials and methods

2.1. Ammonium bicarbonate, lime and pathogens

Ammonium bicarbonate and lime were purchased from the Shanghai JiuYi Chemical Reagent Co., Ltd., Shanghai, China. The pathogens, *F. oxysporum* f. sp. *cucumerinum* (FOC), *F. oxysporum* f. sp. *niveum* (FON) (coded NJAUS-1) and *F. oxysporum* f. sp. *melonis* (FOM), used in the experiments were obtained from the Jiangsu Key Laboratory for Organic Solid Waste Utilization at the Nanjing Agriculture University (Nanjing, China). FOC was isolated from infected cucumber in a field, FON was isolated from infected watermelon in a greenhouse plot and FOM was isolated from infected melon in a greenhouse plot.

2.2. Assays for the destructive effects of ammonium bicarbonate on F. oxysporum mycelia

Plates containing two ammonium bicarbonate concentrations (0.02 g and 0.1 g DW dissolved in 1 ml deionized water each) were covered with potato dextrose agar (PDA) plates (as the top plate) with a 5-mm diameter mycelial plug of the pathogen in the center of each plate to test any destructive effect on the fungus. The two plates were sealed and incubated at 28 °C in the dark for 3 days. Each treatment was replicated three times. To obtain scanning electron microscope (SEM) images, the fungal colonies were obtained from the media (5 mm × 5 mm) using a double-sided blade, transferred to 24-well PVC micro-titer plates and fixed overnight in a 0.075 M phosphate buffer solution containing 2.5% (w/v) glutaraldehyde. Next, the samples were rinsed twice using 0.075 M phosphate buffer for 15 min each, followed by successive 15-min dehydrations in a graded series of ethanol (30, 50, 70, 80, 90, 100 and 100%) before treating with 75 and 100% tert-butanol for 15 min each. All samples were dried in a drier using vacuum sublimation and were coated for 2.5 min with 10 mÅ of gold–palladium before examination with the scanning electron microscope (SEM, S-4800, FESEM, Hitachi, Japan).

2.3. Assays of the antifungal effects of ammonium bicarbonate as a fumigant

To determine the antifungal effects of different concentrations of ammonium bicarbonate as a fumigant on FOC, FON and FOM populations, a cup experiment without plants was set up and four concentrations (1, 2, 3 and 4 g kg^{-1} DW) were applied to the soils (0.1 kg DW each) in 350-ml (plastic) tissue culture bottles. The soil for the cup experiment was collected from three fields that had been continuously cropped with watermelon, cucumber and melon, respectively, for more than 2 years at the Nanjing Institute of Vegetable Science in Nanjing, China. The soil of the three fields were silty and classified as a yellow brown soil and soil characteristics of the three fields are shown in Table S1. The FON, FOC, and FOM population in the soil from watermelon field, cucumber field, and melon field, respectively, was 1×10^4 CFU g^{-1} DW, 2×10^4 CFU g^{-1} DW and 5×10^3 CFU g^{-1} DW, respectively. Spores from different pathogens was added to the corresponding soils to obtain inoculum densities of approximately 5×10^5 CFU g^{-1} DW. These spores were collected as described by Ling et al. (2011), as follows. First, the conidia of FOC, FON and FOM were prepared by growing plate cultures on PDA at 28 °C for 10 d in the dark to induce sporulation. The plates were drenched with sterile distilled water, and the spores were carefully freed from the culture surface using a fine artist's brush. Next, the suspension was filtered through three layers of sterile cheesecloth to eliminate mycelial fragments. The conidia concentrations were determined using a hemacytometer. During the cup experiment, the water content of the soil was maintained between 40 and 60%. Each treatment was composed of three blocks, with each block containing three cups. Soil in each cup was mixed thoroughly, sealed and incubated at room temperature for 7 days. Three sub-samples were collected and combined to form a composite sample (approximately 200 g) for each cup, and